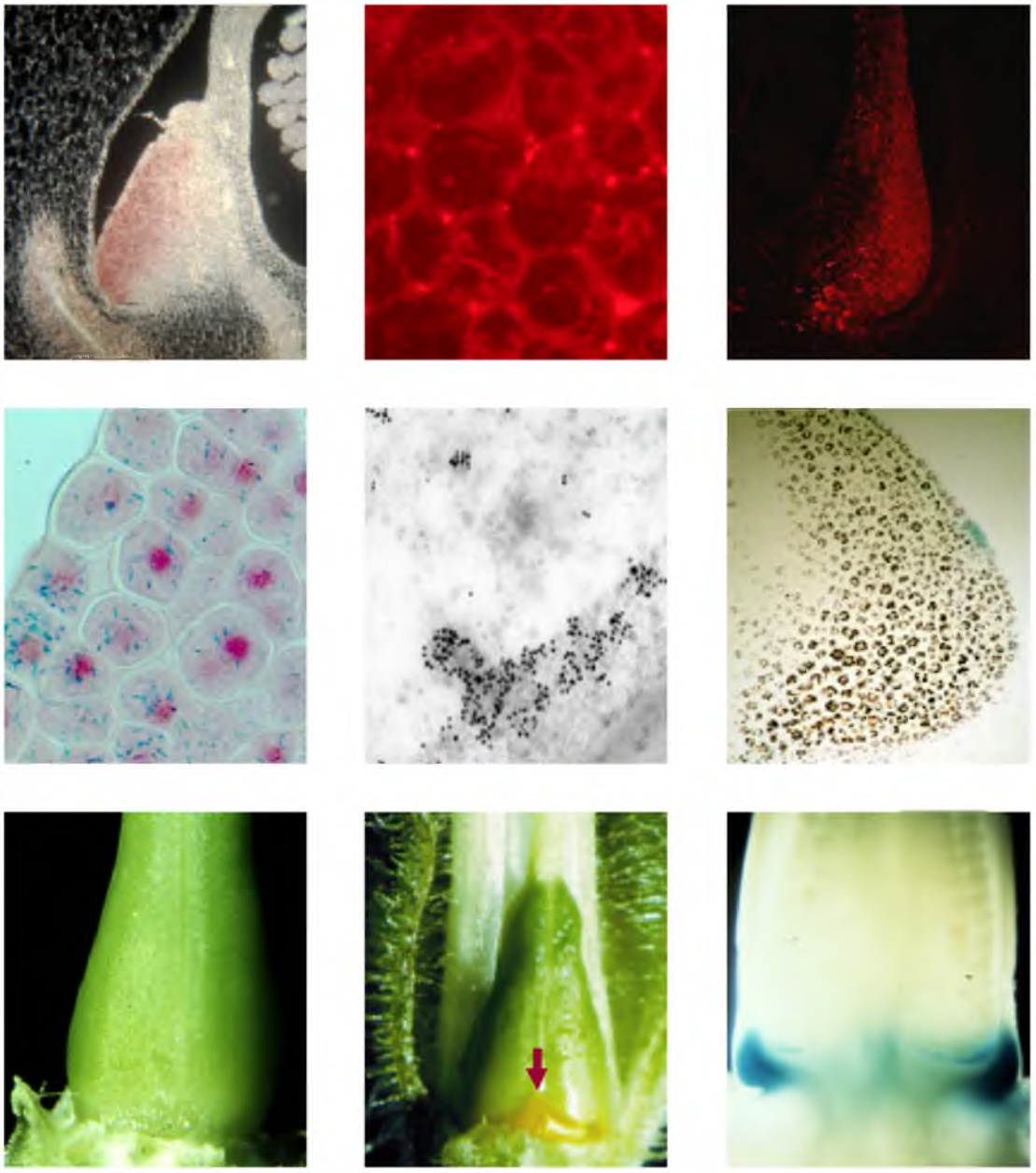
NEC1, a regulator of nectary development and nectar production in *Petunia hybrida*



Ya-Xin Ge

NEC1, a regulator of nectary development and nectar production in *Petunia hybrida*

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Cover Description:

In *Petunia hybrida*, nectaries are located around the base of the pistil (arrow) inside the flower. Localization of *NEC1* mRNA in nectaries by *in situ* hybridization, showed that high levels of *NEC1* transcripts were predominantly observed in the outer nectary parenchyma cells. GUS activity driven by the *NEC1* promoter showed a high level of expression in nectaries. Longitudinal sections through the nectaries showed that GUS activity was present throughout all the nectary parenchyma cells. The pattern of GUS expression appeared to follow the temporal events of starch hydrolysis. Immunolocalization showed that NEC1 protein was present in all nectary cells, the outer parenchyma cells showing the highest concentration. Sub-cellular localization of NEC1 protein showed that specific labeling was present around the plasmalemma and in small vesicles that appeared to fuse with the plasmalemma, followed by nectar secretion.

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NEC1, a regulator of nectary development and nectar production in *Petunia hybrida*

Een wetenschappelijke proeve op het gebied

van de Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

Ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
woensdag 18 april 2001,
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door

Ya-Xin Ge

Geboren op 10 november 1959 te Hebei (Volksrepubliek China)

Promotor:	Prof. Dr. G. J. Wullems
Co-promotor:	Dr. J. Creemers-Molenaar (Plant Research International BV)
Manuscriptcommissie:	Prof. Dr. J.N.M. Mol (Vrije Universiteit, Amsterdam) Dr. M. van Lookeren Campagne (Aventis Crop Science, Gent)

Preface

This thesis was realized with direct and indirect help from many people, to whom I owe many thanks.

First of all, I would like to express my sincere thanks and indebtedness to my promotor, Prof. Dr. George J. Wullems, for giving me the opportunity to carry out research for my Ph.D. degree in the Department of Molecular Plant Physiology, the University of Nijmegen. His valuable guidance, critical comments and suggestions for experiments and for improving my manuscripts were very important and helpful to me. I also wish to express my thanks to Mrs. Yvonne Wullems for her kind hospitality.

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I am grateful to many people from my home institute, Hebei Academy of Agriculture and Forestry Sciences. I am indebted to the president of Hebei Academy of Agricultural and Forestry Sciences, Prof. Jiankun Wei, and to the directors of the institute, Shiguang Sun and Xiuqin Feng for their support and understanding. I owe a great deal of gratitude to colleagues in our group, especially to Prof. Dr. Jiangfang Wei for taking over all my responsibilities and work when I was in The Netherlands and to Chunhong Ma and Shiping Cui for all their friendship and help.

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Finally, I greatly appreciate my husband, Guangming and our daughter, Yunfei, for their love, continuous support and understanding throughout my whole Ph.D. period. Also, I am greatly indebted to my aunt Lifang Ge, my brothers Maosheng Ge and Xinsheng Ge, and all our family in China for their encouragement and taking care of Yunfei.

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Yaxin Ge

Contents

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Nectary development is one of the striking events in the life cycle of higher plants. Despite extensive descriptions in the literature on the morphology, ultrastructure, phylogenetic distribution and pollination biology of nectaries, little is known about genes directing their development and function.

The work described in this thesis contributes to solve this gap by comprising the competent cloning, characterization, expression and functional analyses of a nectary-specific gene in *Petunia hybrida*. Nectar secreted from floral nectaries is the main floral reward for pollinating insects, and nectar quality is the key value for selecting plants that are more attractive to pollinators. Genetic regulation of nectary development and modification of nectar composition, the new biotechnological approach, will allow to change e.g. nectar sugar type and concentration, making plants more attractive to pollinators.

In this chapter, as an introduction to this thesis, a general review is given including a description of nectary morphology, nectar secretion and molecular regulation of nectary development.

Nectary location and ultrastructure

Nectaries are nectar-secreting organs of plants, located in the nuptial flowers (floral nectaries) or on vegetative structures outside the flowers (extrafloral nectaries), both of which excrete nectar (Fahn, 1988; Rogers, 1985). The mutual role of floral nectar in plant pollination and insect feeding is well known, however the function of the extrafloral nectary has been debated. Undoubtedly extrafloral nectaries serve some useful biological function, e.g. ants, which may have a role in plant defense, are the most abundant extrafloral nectary visitors (Bentley, 1977; Rogers, 1985).

Among different plant species, the floral nectaries do not have a fixed position on plant surfaces (Link, 1992). Anatomically, the nectaries are attached to the base of the filament, the petal, the ovary or other floral organs (Brackenbury, 1995; Galetto, 1995; Link, 1992). They are positioned on the surface of the organ bearing them, form an outgrowth on the organ, such as the nectaries of *Petunia* and Brassicaceae or may be sunken deeply inside the organ, e.g., cotton nectaries.

Electron microscopic studies revealed a number of ultrastructural characteristics of nectary cells. Often, numerous plasmodesmata exist between the nectariferous cells (Fahn and Rachmilevitz, 1970; Fahn and Benouaiche, 1979), where vesicles occurring in the cytoplasm seem to be in contact with the plasmalemma (Fahn, 1979; Eleftherious and Hall, 1983). In addition, the endoplasmatic reticulum (ER) is highly developed and its cisternae are often arranged in stacks. At the stage of secretion, the ER is associated with vesicles (Fahn and Rachmilevitz, 1970; Fahn and Benouaiche, 1979; Mohan and Inamdar, 1986; Figueiredo and Pais, 1992). A high number of active

dictyosomes and mitochondria are present in nectary cells (Fahn and Benouaiche, 1979; Christ and Schenpf, 1985; Marginson *et al.*, 1985) during the stage of secretion. Plastids occur in varying numbers, and most of these contain starch grains. In some plants, just before secretion, the amount of starch grains is very large (Darkee *et al.*, 1981) and it declines at the stage of secretion (Zer and Fahn, 1992).

In *Petunia hybrida*, one pair of nectaries connected with a ring of nectariferous tissue is located around the base of the pistil inside the flower (Figure 1B, C). Nectary tissue consists of parenchyma cells, underlying a single epidermal cell layer and containing bundles of vascular tissue (Figure 1D, E). Sugar and starch accumulate during the development of nectaries. After anthesis, nectary cells

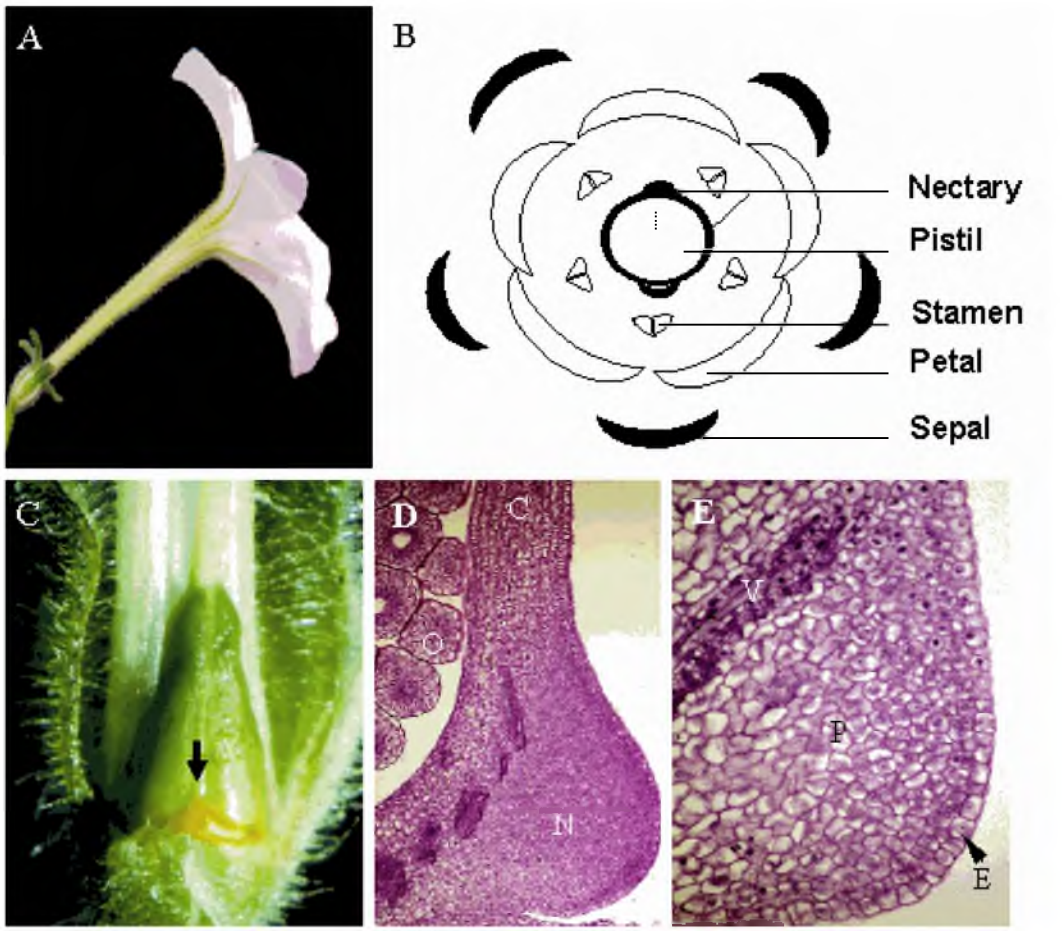

Figure 1. Floral nectaries of *Petunia hybrida*. Flower **(A)**; Floral diagram showing the location of the nectaries **(B)**; Nectary at the base of pistil (arrow) **(C)** and longitudinal sections showing nectaries (N), vascular bundles (V), nectary parenchyma (P) and epidermis (E) **(D, E)**. O, ovule; C, carpel.

contain large vacuoles and show high nectar secretion, whereas the majority of starch has been hydrolyzed. Nectaries are white in younger flower buds (<4cm) that do not

secrete nectar, and become light yellow, yellow and orange during flower development, while nectar secretion increases concomitantly.

Nectary morphology and nectar secretion have been extensively studied, particularly in Brassicaceae. In *Brassica napus*, each flower contains two pairs of nectaries with different nectar-producing capacities. The lateral nectaries have an extensive supply of phloem, and they produce most of the flower's nectar (Davis *et al.*, 1976). The median nectaries are supplied by limited phloem and produce relatively little nectar (Davis *et al.*, 1986, 1994).

Nectar composition has been studied in many flowering species. Nectar contains considerable quantities of sucrose, fructose and glucose, the concentration and ratio of the different sugars varying between plant species. The observed differences in nectar composition may reflect pollinator preferences (Baker and Baker, 1975, 1982). The role of nectar composition in pollinator attraction will be further discussed in chapter 2, as a review.

Nectar secretion mechanisms

The structure and ultrastructure of nectaries in relation to nectar secretion has been described for several plant species. The cells in nectaries, constituting the nectariferous tissue, usually contain branches of very well developed vascular bundles, which contain a high proportion of phloem elements. Being a specialized sink organ, the nectaries are supplied with sucrose by phloem unloading (Davis *et al.*, 1986; Zer and Fahn, 1992).

Nectar is secreted from nectaries either directly by epidermal cells or trichomes, or indirectly by cells of the subepidermal nectariferous tissue. In the latter case, nectar is secreted into intercellular spaces that are connected to the stomata (Fahn, 1988; Davis and Gunning, 1993). It has been shown that the number of nectary stomata itself is not related to the quantity of nectar secreted (Teuber *et al.*, 1980; Davis and Gunning, 1993; Petanidou, 1995). There is, however, a positive relation between the quantity of nectar secretion and nectary size (Dafni *et al.*, 1988).

The mechanisms of sugar accumulation and nectar secretion have been described (Fahn *et al.*, 1979b). Figure 2 presents a schematic diagram of the main processes leading to nectar secretion. Sugar transport to the nectaries is achieved by active transport mechanisms and a combination of osmotic and chemical gradients. In many plants, sucrose is converted to glucose and fructose in the nectaries, resulting in hexose dominant nectar. Part of the hexoses is converted to starch, which is hydrolyzed prior to anthesis and nectar secretion. Cell to cell transport of

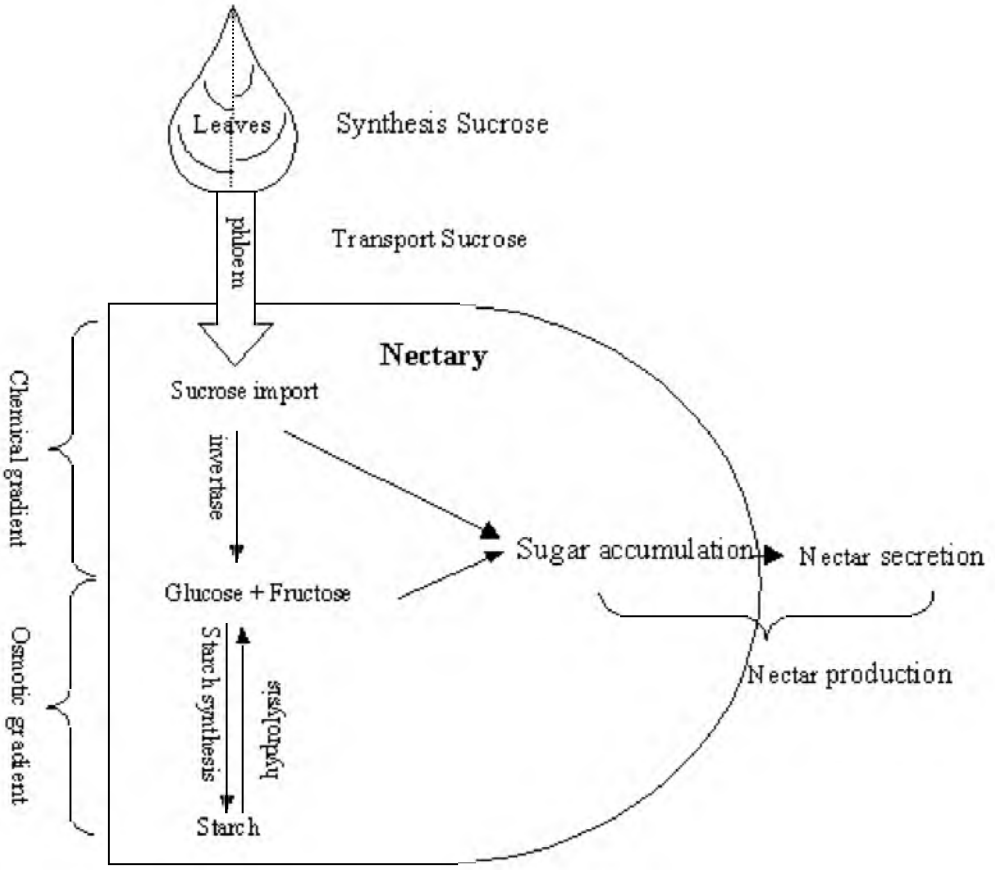

Figure 2. Sugar transport into nectary cells and nectar production. Sucrose import to nectary cells is facilitated 1) by a chemical gradient, because sucrose is converted to hexoses (glucose/fructose), 2) by an osmotic gradient, because glucose is converted to starch and, 3) by active transport by sugar transporters. Nectar production is defined as the combination of sugar accumulation and nectar secretion.

nectar in the nectary parenchyma tissue is mainly symplastic, as indicated by the presence of many plasmodesmata between these cells (Fahn *et al.*, 1979). An overview of possible ways of sugar transport into a secretory cell and of nectar elimination from this cell, is diagrammatically presented in Figure 3. The secretion of nectar follows fusion of vesicles that originate from the ER or Golgi with the plasmalemma (Fahn, 1979)

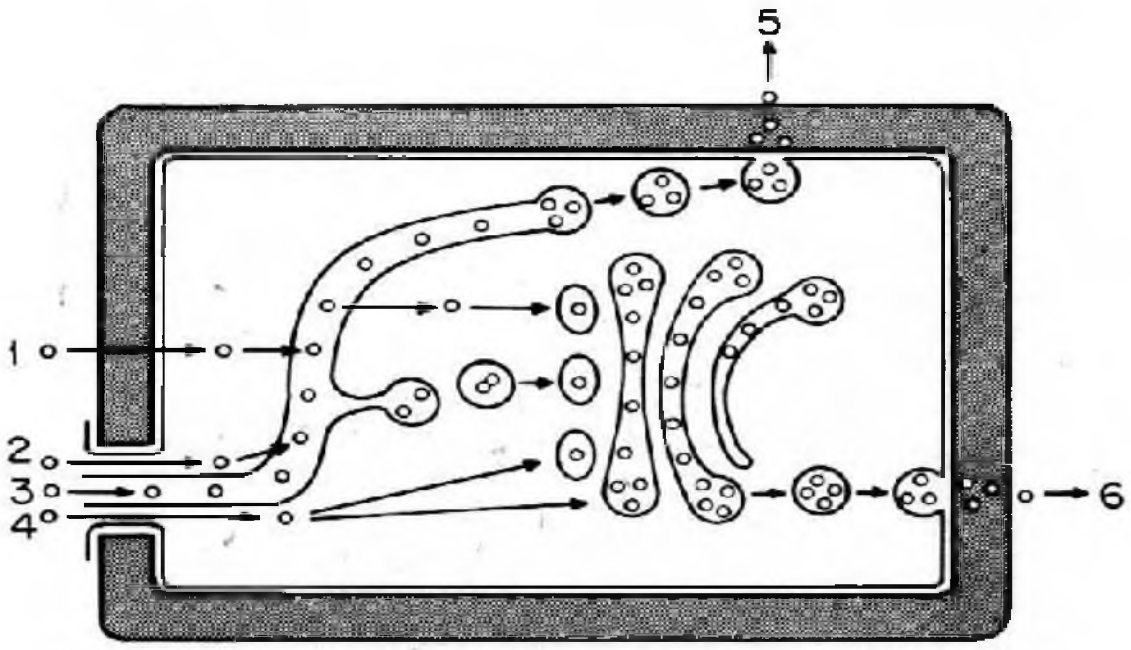

Figure 3. Diagrammatic presentation of possible ways of sugar transport into a secretory cell, and nectar secretion from this cell (based on Fahn, 1979 b, *Amer. J. Bot.*66 (8): 984). (1) Molecular transport through plasmalemma to the ER; (2) Transport through the cytoplasmic annulus of a plasmodesma into the protoplast and entering into the ER by membrane transport; (3) entrance to the ER of the secretory cell through desmotuble of a plasmodesma; (4) as in (2), but entering a Golgi body; (5) secretion of nectar by fusion of vesicles which originated from the ER with the plasmalemma; (6) secretion of nectar by fusion of Golgi vesicles with the plasmalemma.

Molecular regulation of nectary development in *Petunia hybrida*

To date, approximately 30 species of the genus *Petunia* (Solanaceae) have been described. The ancestors of *Petunia hybrida* are *Petunia axillaris* and *Petunia inflata* (Colombo *et al.*, 1997a; Sink, 1984).

Petunia hybrida is highly suitable as a model species for gene tagging, because of the presence of well-characterized transposable elements that can jump in and out of genes. Simple PCR techniques can be applied to identify transposon-induced mutations in a gene of interest, or to tag a gene that contains a transposable element. In addition, much is already known about the molecular regulation of flower development, and has been described for sepals, petals, anthers and carpels (Angenent *et al*, 1992, 1993, 1995a, 1995b; Purugganan *et al*, 1995).

The molecular control of nectary development and nectar secretion has not yet been elucidated. There have been, however, some reports that described genes which are expressed in nectaries, including *CRT*, *CRABS CLAW* and *AGL1* genes in *Arabidopsis thaliana* (Bowman and Smyth, 1999; Flanagan *et al.*, 1996; Nelson *et al.*, 1997), a *myb* gene in *Antirrhinum* (Jackson *et al.*, 1991) and *NTR1* gene in *Brassica campestris* L. *ssp. pekinensis* (Song *et al.*, 2000). However, the function of these genes in nectary development and nectar secretion was not clear. Recently, as described in this thesis, the molecular regulation of nectary development in *Petunia hybrida* has been investigated (Ge *et al.* 2000, 2001a, b). Briefly, *NEC1*, a nectary-specific gene was cloned, which is involved in nectar production and nectar secretion. It was shown that *NEC1* is predominantly expressed in nectaries of *Petunia hybrida* and the nectar-secreting lateral nectaries of *Brassica napus*, and weakly in stamens, where it is localized in stomium cells and at the top of the filament. The expression is highest at the stage after anthesis when active nectar secretion takes place. Co-suppression and transposon mutagenesis experiments resulted in partial down-regulation of the *NEC1* transcript in transgenic plants, revealing disturbed in vitro pollen germination and an "early open anther" phenotype. Antisense expression of *NEC1* resulted in complete down-regulation of *NEC1* and a distinguishable nectary phenotype, characterized as impaired nectary development and strongly reduced nectar production in *Petunia hybrida*. *NEC1* promoter activity was demonstrated in plants transformed with gene constructs that comprised the GUS gene or the *Barnase* gene, downstream of the *NEC1* promoter. Nectary-specific gene expression of *Barnase* in *Petunia* resulted in cell ablation at an early stage of nectary development, yielding nectariless flowers.

Aim and scope of the thesis

The main objectives of the research presented in this thesis are to elucidate nectary-specific gene expression in relation to nectary function by cloning and characterizing nectary-specific genes. The ultimate aim is to use such genes to modify nectar composition of floral nectaries in order to make flowers more attractive to honeybees.

Chapter 2 gives an overview of floral nectar composition in relation to pollinating insects.

In chapter 3 results are reported on cloning and expression of the *NEC1* gene from *Petunia hybrida*. *NEC1* is highly expressed in nectaries of *Petunia hybrida*, in the lateral nectaries of *Brassica napus* and very localized in the stamens of both species. Gene expression was studied by Northern blot hybridization, RT-PCR and *in situ* hybridization. In addition, Western blotting and immunolocalization were applied to reveal the pattern of NEC1 protein expression. GUS activity under the control of the *NEC1* promoter showed the tissue specificity and exact timing of the

NEC1 gene expression. The phenotype, due to the ectopic expression of *NEC1*, is also described in this chapter.

In chapter 4, the "early open anther" phenotype is described. This phenotype was obtained by gene silencing of *NEC1* by co-suppression and transposon mutagenesis. The results of down-regulation are discussed in relation to the presence of two highly homologous genes, *NEC1* and *NEC2*, in *Petunia hybrida*.

Chapter 5 describes the mutant nectary phenotype that resulted from antisense suppression of *NEC1* under the control of the *NEC1* promoter. Furthermore, the effect of *Barnase* under the control of the *NEC1* promoter is presented. A model for the molecular regulation of nectar production in *Petunia hybrida* is presented and, the practical implications of nectariless plants are discussed.

Finally, in chapter 6, a general discussion is presented on the possible roles of *NEC1* in nectar production and sugar metabolism.

CHAPTER 2

FLORAL NECTAR IN RELATION TO POLLINATING INSECTS:

A REVIEW

Table of contents

Introduction

One of the longest partnerships in biological history is the relationship between plants and insects. Co-evolution of flowers and pollinators has led to an amazing variety of floral forms and pollinator's tongues. It is a fair exchange, but not a robbery that the insects could be duped in rendering a service, i.e. pollination, as long as they are offered a suitable reward (Baker and Baker, 1983a; Brackenbury, 1995; Kampny, 1995).

Flowers attract insects by providing visual and olfactory stimuli to insects, which seek food rewards from flowers. In return, the insects transport pollen to flowers which they subsequently visit, and thus bring about pollination for sexual plant reproduction (Kevan, 1983). After a flower is pollinated, the petals wither and senesce, and nectar production cease soon afterwards. In unpollinated flowers, nectar secretion persists longer than the usual period (Free, 1970).

Floral nectars are known to have an important ecological role in attracting pollinating insects. Nectar mainly consists of a sugar solution, glucose, fructose, and sucrose being the main sugars (Fahn, 1979; Vogel, 1983). Nectar is obviously a reward for pollinating insects in many species (Meve and Liede, 1994). Many flowers appear to have certain markings that seem to guide or direct nectar feeders to the nectar source. Apart from visual attraction, odors are the most important cues for attracting and orientating pollinators. The particular kind of odor actually allows the insects to discriminate the flowers and release behavioral reactions that are necessary for pollination (Harrewijn *et al.*, 1995).

There is a wide variety of flowers and insects, and flower-pollinator combinations are often very precise. Thus, the insects recognize the flowers that provide their needs, and the flowers, in turn, attract the insects that fit to their needs (Kevan, 1983). "Nectar sugar ratio" shows a close relationship with the type of pollinator that visits the flowers (Baker and Baker, 1990). There is also a general agreement between sugar ratios and the nature of the principal pollinators (Baker and Baker, 1983b).

Floral nectar constituents

The nectaries, or nectariferous tissues that secrete nectar, are found in many parts of the flower including the receptacle, petals, sepals, the base of the filament and the pistil (Free, 1970; Link, 1992; Brackenbury, 1995; Galetto, 1995).

The composition of nectar has been widely studied. Nectar contains mostly sugar, but small amounts of other substances contribute to its aroma and to the characteristics of the honey prepared from it. The three main sugars of nectar are sucrose, fructose and glucose. The minor sugars present in various species include

maltose, raffinose, melibiose, trehalose and melezitose (Shuel, 1955; Percival, 1965; Free, 1970; Fahn, 1979; Luttge, 1977; Baker and Baker, 1983b; Vogel, 1983). All twenty normal amino acids found in protein have been identified in various nectars (Baker and Baker, 1973, 1986; Carter, *et al.*, 1999). Other substances reported in nectar include organic acid, terpenes, alkaloids, flavonoids, glycosides, vitamins, phenolics, and oils (Baker and Baker, 1975; Roshchina and Roshchina, 1993; Carter, *et al.*, 1999). A limited array of proteins is present in the floral nectars of leek (Peumans *et al.*, 1997) and tobacco (Carter, *et al.*, 1999).

The ratios of sugars, which nectar has a sucrose/hexose ratio by weight of sucrose, glucose, and fructose, differ markedly between species. There is a general constancy within a species in classifying the nectar in one of four classes: hexose-dominant (nectars with a sucrose/hexose ratio of less than 0.1), hexose-rich (nectars with ratios between 0.1 and 0.499), sucrose-rich (nectars with ratios between 0.5 and 0.999) and sucrose dominant (those with ratios of more than 0.999) (Baker and Baker, 1983b, 1990). The constancy of nectar sugar ratio reflects its resistance against environmental variation (Baker and Baker, 1983b). According to sugar content, the nectars of angiosperms are usually divided into three groups: sucrose prevalent, glucose and fructose prevalent, and an equal amount of sucrose, glucose, and fructose (Roshchina and Roshchina, 1993).

Although the proportion of the different sugars present tends to remain constant in a given species (Wykes, 1952a, Percival, 1961; Free, 1970), there may be large differences in the average sugar concentration of nectar in different species and different varieties of the same species (Pervival, 1965; Free, 1970). The sugar concentrations vary greatly depending on the type and the location of the nectary (Roshchina and Roshchina, 1993). Nectar of flowers with deep corolla tubes and protected nectaries contains mostly sucrose with small amounts of glucose and fructose, whereas the nectar of shallow flowers with unprotected nectaries contains mostly glucose and fructose, but little sucrose (Percival, 1961). Nectar secretion is influenced by the maturation of the stigma and stamens, and also often by the age of the flower (Shuel, 1961; Free, 1970). The rate of nectar production and the sugar concentration can vary independently with the age of the flowers. Most plants also show day-to-day changes in peaks of nectar secretion and sugar concentration. The visit by bees and other nectar-gathering insects may also increase nectar secretion (Wykes, 1953; Free, 1970). Flowers pollinated by high-energy requiring animals, for example, bats, hawkmoths and birds, produce significantly more nectar than flowers pollinated by low-energy requiring animals, such as butterflies, bees and flies (Cruden *et al.*, 1983). Day-flowering plants secrete less nectar at night, while plants having nocturnal pollinators secrete less during the day (Proctor and Yeo, 1973; Brackenbury, 1995). The concentration of nectar can be increased by evaporation, or decreased by dilution with rain (Proctor and Yeo, 1973). In hot weather, the rate of evaporation can

exceed the rate of secretion so that sugar crystallizes out on the surface of the nectaries. If insects do not visit the flower, nectar accumulates in globules on the surface of nectaries (Brackenbury, 1995).

Nectar as an insect food source

Nectar together with pollen constitutes the two primary pollinator attractants. Flowers produce two of the most vital ingredients in an insect's diet: carbohydrate and protein (Brackenbury, 1995). Carbohydrate, the source of energy, is the principal food requirement of adult winged insects, and is the main constituent of nectar. Pollen undoubtedly contributes towards the protein requirements.

Bees recognize flowers by their color, shape and odor, and are attracted to flowers for collecting nectar and pollen (Free, 1970; Kevan, 1983), and especially to nectar sugars (Rabinowitch *et al.*, 1993).

Nectars secreted from nectaries, which contain sucrose only, or mixtures in various proportions of sucrose, glucose, fructose, is virtually a pure sugar. The nectar sugars are the carbohydrate reward for pollinating insects, which are required by the plants for sexual reproduction (Proctor and Yeo, 1973). Sucrose (cane sugar) is a disaccharide, which can be converted into equal parts of two monosaccharides, glucose and fructose, by the action of the enzyme invertase. Nectar is not the only natural source of sugar in plants, since there are also sugars in fruits, which are rich in fructose. However, these sugars are often locked up inside the more complex molecules, such as plant starches and cellulose. Insects that eat such complex sugars can render monosaccharides from these in the insect's gut, provided it possesses the appropriate enzymes (Brackenbury, 1995). Nectar is a pure sugar, and is therefore a concentrated source of energy for insects. Once eaten, it is rapidly absorbed and digested, and ready for use by the tissues. Nectar is the ideal food source for insects like bees, butterflies and moths that spend a lot of time on flying by means of wings and generate a high demand for energy. Nectar-feeding insects need to spend a lot of time flying between flowers simply to gain enough food to suffice for their needs (Brackenbury, 1995).

Floral nectar characteristics and pollinator types

The nectar significance in relation to pollination, taxonomy, and evolution has been studied extensively (Baker and Baker, 1975, 1983b, 1990). Because nectar is the only floral reward that functions solely as such, it is likely to be subject of a selection pressure imposed by pollinators (Baker and Baker, 1983b; Petanidou *et al.*, 1996). As a result, closely related plants with different pollinators have different nectars (Baker and Baker, 1983b). In other words, nectar and its characteristics define the type of

plant-pollinator interactions (Brink and de Wet, 1980).

As presented above, sucrose, glucose, and fructose, are the bases of the energy reward that flower-visitors receive when they take nectar from flowers. Both the sugar types and concentrations are ecologically important. The different levels of amino acids that occur in distinct nectars suggest that they have an adaptive significance. In fact, nectars that are collected by butterflies, noctilucas, wasps and dung flies have substantially higher levels of amino acids than most nectars of bee-, sphingid-, bird- and bat- flowers. Even when present in traces, amino acids can act as phago-stimulants or taste-determinants (Vogel, 1983).

Nectar sugar ratios show a close relationship with the types of pollinators that visit the flowers. Similarities exist in sugar-ratios between plants with the same pollinator type, even if the plants are taxonomically unrelated (Baker and Baker, 1983b, 1990). A general trend shows that species in the same family, and plants pollinated by the same class of pollination vectors, have a similar sugar ratio. Nectars that are rich in sucrose are secreted by flowers pollinated by lepidoptera as well as by those pollinated by hummingbirds or large bees (Baker and Baker, 1990). Flowers pollinated by small bees, passerine birds, or neotropical bats are rich in hexoses (Percival, 1961; Hainsworth and Wolf, 1976; Stiles, 1976; Baker and Baker, 1983b; Scogin and Freeman, 1984; Baker and Baker 1990).

It is very remarkable that bee-pollinated flowers, in particular, deep flowers visited by long-tongued bees, have sucrose-dominant nectars, whereas open flowers visited by short-tongued bees have hexose-dominant nectars (Baker and Baker, 1983b).

Wykes (1952b) found that honeybees preferred solutions of single sugars in the following descending order: sucrose, glucose, maltose, and fructose. The mixture of equal parts of sucrose, glucose and fructose was preferred to a solution of any single sugar of the same concentration or to a mixture of these sugars in different proportions. This latter finding is surprising as few nectars have equal proportions of the three main sugars and most are either sucrose-dominated or fructose-glucose dominated (Free, 1970).

The concentration of sugar in the nectar of plants also reflects the gustatory preferences or physical limitations of the pollinators (Cruden *et al.*, 1983). There is evidence that bee- and fly- pollinated flowers tend to secrete highly concentrated nectars, while hawkmoth-, bird- and bat-flowers have thin solutions (Vogel, 1983). The nectars of bee-pollinated flowers tend to have higher sugar concentrations than the nectars of flowers pollinated by other insects (Baker, 1975). Honeybees, at least, prefer sugar concentrations of 30 to 50 percent (Waller, 1972), whereas nectars of most butterfly-pollinated flowers fall within the range of 15 to 25 percent (Watt *et al.*, 1974).

The flower attractiveness is different between species and varieties, along with different average sugar concentrations, even within a single flower, and especially in

shallow open ones. The sugar concentration is subjected to a considerable fluctuation as a result of exposure to wind and rain, and changes in temperature and relative humidity. Hence, the attractiveness of a species may differ at different times of the day and at different stages of flowering (Free, 1970).

In addition to friends among insects, flowers have also enemies, including the destructive caterpillars, such as hawk moths (Brackenbury, 1995). Nectars may even contain toxic alkaloids, non-protein amino acids, or other substances like phenolic substances (Baker 1977, 1978; Baker and Baker, 1975, 1983a). Most Lepidoptera will not tolerate alkaloids in nectar, but bees (and presumably other insects as well) will continue to pollinate flowers having alkaloids in floral nectar (Baker and Baker, 1979; Rogers, 1985).

Clearly, what is needed for a better view on the plant-pollinator relation is a series of studies of pollinator rewards on an ecosystem basis, rather than just taking each flower species and each visitor species in isolation (Baker and Baker, 1983b). Although, the honeybee is very important as pollinator of contemporary plants, it could not be a representative of the bees as a whole, so that generalizations about bee preferences and needs should not be based on honeybees alone (Baker and Baker, 1983b).

Pollination requirement of crops

Insects play a vital role in the pollination of many plants, including some of our most important cultivated crops. Plant species of economic importance produce fruits or seeds by either self-pollination or cross-pollination. Many of the wild plants in nature and crop plants for agriculture and horticulture are cross-pollinated. Moreover, self-fertile plants may produce more fruits, or seeds of better quality, when cross-pollinated than when self-pollinated (Free, 1970).

Wind is the principal pollinating agent of agricultural grasses and a few other species, whereas most agricultural and horticultural crops depend on cross-pollination by insects for seed-set and the production of fruits. Pollinating insects are primarily interested in collecting nectar, but the process of nectar collection can result in cross-pollination. Cross-pollination within the cultivar ensures the best seed development. The utilization of plants with high nectar quality for semi-natural lands and agriculture can overcome the decline of biodiversity of pollinating insects and can improve the quality and the yield of seeds and fruits.

The most important pollinating insects are solitary bees, bumblebees and honeybees. Bees with sufficient body hairs are the most specialized pollen vectors and depend almost entirely on the pollen and nectar of flowers. Furthermore, bees consistently forage and make several visits to flowers to obtain sufficient food for their young bees, and therefore they are extremely effective pollinators (Free, 1970; Proctor and Yeo, 1973).

Honeybee populations can be artificially managed much easier than other bees, and hence they are of utmost importance as agricultural pollinators. Bumblebees may be even more efficient pollinators than honeybees, since they work faster and for longer hours, and carry greater loads of pollen (Frankel and Galun, 1977).

Brassica, tomato (*Solanum Lycopersicon*) and cotton (*Gossypium spp.*) are major economic crops and the most extensively grown species. Most of the *Brassica* species are self-incompatible to some extent, but this varies with the species, varieties and even with the age of plants (Free, 1970). Although the tomato is self-fertile, it can not rely on self-pollination, as it usually requires some disturbance of the flowers. Without this, there may be too few flowers pollinated to give the maximum fruit-yield, and too few ovules fertilized in each flower to give well-formed fruits. The same variety of the tomato in an area where it is pollinated by insects, is at least twice as efficient (Proctor and Yeo, 1973).

Cotton (*Gossypium spp.*) is usually referred to as a partially cross-pollinated crop. The benefits derived by cotton from insect pollination have been cited by numerous workers. Briefly, cotton benefits from bee pollination in terms of greater and higher quality lint and seed production, earliness of harvest, better germination, and improved qualities in the offspring (McGregor, 1976). Especially, hybrid cotton plants, because of hybrid vigor, show increase of yield, lint percentage, lint index and seeds per boll. For large-scale production of true hybrid seeds, one of the parental lines chosen is often male sterile. Pollen, which is too heavy and sticky to be carried by wind, has to be transferred from the male-fertile to the male-sterile plants either by hand-pollination or by insect-pollination. Hand-pollination is labor-intensive, and therefore expensive, while insect pollination is cheap and increases seed quality. In general, efficient pollination of crops results in good seed-set and high fruit quality.

Concluding remarks

Insect pollination is a very efficient pollination system and the most important one for cultivated plants. The majority of crop plants are insect-pollinated. Efficient pollination of crops results in good seed-set and high fruit quality. However, in most hybrid crops, inadequate pollination is one of the major problems in seed production. There is still much to be known about flower pollination and how to make use of the honeybee, wild bees, and other insects to achieve maximum efficiency in agricultural production. At the same time, the information we now have on the subject is not being utilized to the fullest extent for improving yields and quality of many kinds of fruits, vegetables, and field crops. Chapter 6 gives a further discussion on this aspect.

It is clear that nectar is a floral carbohydrate reward for the pollinating insects and nectar characteristics define the type of plant-pollinator interaction. Floral nectar quality is the key value to attract the pollinating insects. The use of attractive plants in semi-natural lands for pollinators will enhance biodiversity of pollinating insects and wild plants. At present, several sugar metabolism genes are available, and investigations aimed at the isolation of nectary-specific genes are being carried out. Genetic modification of nectars, which are more attractive to pollinators but repellent to pests, is of great immediate importance. Therefore, further research on genetic regulation of nectar composition and the effects on insect attraction, is highly desirable.

CHAPTER 3

NEC1, A NOVEL GENE, HIGHLY EXPRESSED IN NECTARY TISSUE OF *PETUNIA HYBRIDA*

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Summary

To study the molecular regulation of nectary development, we cloned *NEC1*, a gene predominantly expressed in nectaries of *Petunia hybrida* by using the differential display RT-PCR technique. The secondary structure of the putative NEC1 protein is reminiscent of a transmembrane protein, indicating that the protein is incorporated in the cell membrane or the cytoplast membrane. Immunolocalization revealed that NEC1 protein is present in the nectaries. Northern blot analyses showed that *NEC1* is highly expressed in nectary tissue and weakly in the stamen. GUS expression driven by the *NEC1* promoter revealed GUS activity in the outer nectary parenchyma cells, the upper part of the filament and the anther stomium. The same expression pattern was observed in *Brassica napus*. GUS expression was observed as blue spots on the surface of very young nectaries that do not secrete nectar and do accumulate starch. GUS expression was the highest in open flowers in which active secretion of nectar and starch hydrolysis had taken place. Ectopic expression of *NEC1* resulted in transgenic plants that displayed a phenotype with leaves having 3-4 times more phloem bundles in mid veins than the wild type *Petunia*. The possible role of *NEC1* gene in sugar metabolism and nectar secretion is discussed.

Keywords: nectary, *NEC1* gene, anther stomium, nectar secretion, *Petunia hybrida*, *Brassica napus*.

Introduction

Nectaries are nectar-producing organs that are located inside (floral) or outside them (extrafloral) (Fahn, 1988; Rogers, 1985). Nectaries are attached to the base of the filament (Link, 1992), the ovary or other floral organs (Galetto, 1995). Nectar is composed of sugars, but small amounts of other substances can also be present (Fahn, 1979; Vogel, 1983). The main sugars found in nectar are sucrose, glucose and fructose. The ratio between the different sugars, the sugar concentration and the volume of nectar production differ markedly between species (Baker and Baker, 1982). Because nectar's function is the floral reward for pollinating insects, the observed differences in nectar composition probably reflect pollinator preferences (Baker and Baker, 1975).

Previous investigations were mainly focused on nectary morphology (Davis *et al.*, 1996; Fahn, 1988), nectar secretion (Fahn, 1979) and nectar composition (Baker and Baker, 1982). The molecular regulation of nectary development and nectar secretion is poorly understood. Calreticulin (CRT), a calcium-binding protein, is

expressed in nectaries of *Arabidopsis thaliana*, but its function is unclear (Nelson *et al.*, 1997). The *CRABS CLAW* gene appears to be essential for nectary development in *Arabidopsis thaliana* (Bowman and Smyth, 1999). A carpel-specific gene of *Arabidopsis*, *AGL1* is also strongly expressed in the nectaries (Flanagan *et al.*, 1996). In addition, a *myb* gene of *Antirrhinum* showed high expression in nectary tissue (Jackson *et al.*, 1991). The approach of our study is to clone genes that are highly and specifically expressed in nectaries and to study the function of these genes. The ultimate aim is to use these genes for genetic modification of nectary development or nectar composition.

Results

NEC1 encodes a putative membrane spanning protein

By differential display RT-PCR, a nectary-specific cDNA clone was obtained, comprising a 3'terminal fragment of 470 base pairs. Using RACE-PCR, the full-length cDNA of 1204 bp was obtained. The deduced amino acid sequence reveals a protein of 265 amino acid residues (Figure 1A). The putative NEC1 protein shows high homology (47% identity, 72% similarity) with *MtN3*, a *Rhizobium*-induced gene that is involved in nodule development in the legume *Medicago trunculata* (Gamas *et al.*, 1996).

The hydropathy plot of the deduced NEC1 polypeptide is shown in Figure 1B. The putative protein contains seven hydrophobic transmembrane segments. The C-terminus is highly hydrophilic. Highest homology with *MtN3* is found in the N-terminal sequence, the first two and the last two membrane spanning loops. The hydrophilic C-terminal part shows the lowest homology (28% identity, 30% similarity).

NEC1 is strongly expressed in nectaries

RNA and protein expression patterns of *NEC1* were investigated by Northern blot analysis, RT-PCR, *in situ* hybridization and Western blot analysis (Figure 2A-E).
The expression pattern of *NEC1* in *Petunia* was determined by RNA gel blot hybridization, using RNA of leaves, sepals, petals, stamens, pistils and nectaries. Figure 2A shows that *NEC1* expression is exclusively observed in nectaries. After exposure for more than one week, a weak signal was also detected in the stamens (data not shown). The expression of *NEC1* in the stamens was confirmed by RT-PCR on

different floral and vegetative tissues (Figure 2D). In addition, RT-PCR revealed weak expression in the pistils, while after gel blot analysis, a weak signal was also detected in the petals and leaves (Figure 2D).

To analyze the temporal expression of *NEC1*, RNA was isolated from nectaries of different flower stages, as described in Table 1. RNA gel blot analysis shows that mRNA of *NEC1* already accumulated in nectaries of closed flower buds that do not yet secrete nectar. The expression level of *NEC1* slightly increased during nectary and flower development (Figure 2B).

Table 1. Various flower stages used for nectary analysis in *Petunia hybrida*

Stage	Length*	Flower	Anther	Nectary colour	Nectar secretion
1	3-4 cm	closed	closed	white	-
2	5-6 cm	closed	closed	Light yellow	+
3	6- cm	open	closed	yellow	++
4	6- cm	open	open	orange	++

* Flower length is measured from the base of sepals to the end of corolla.

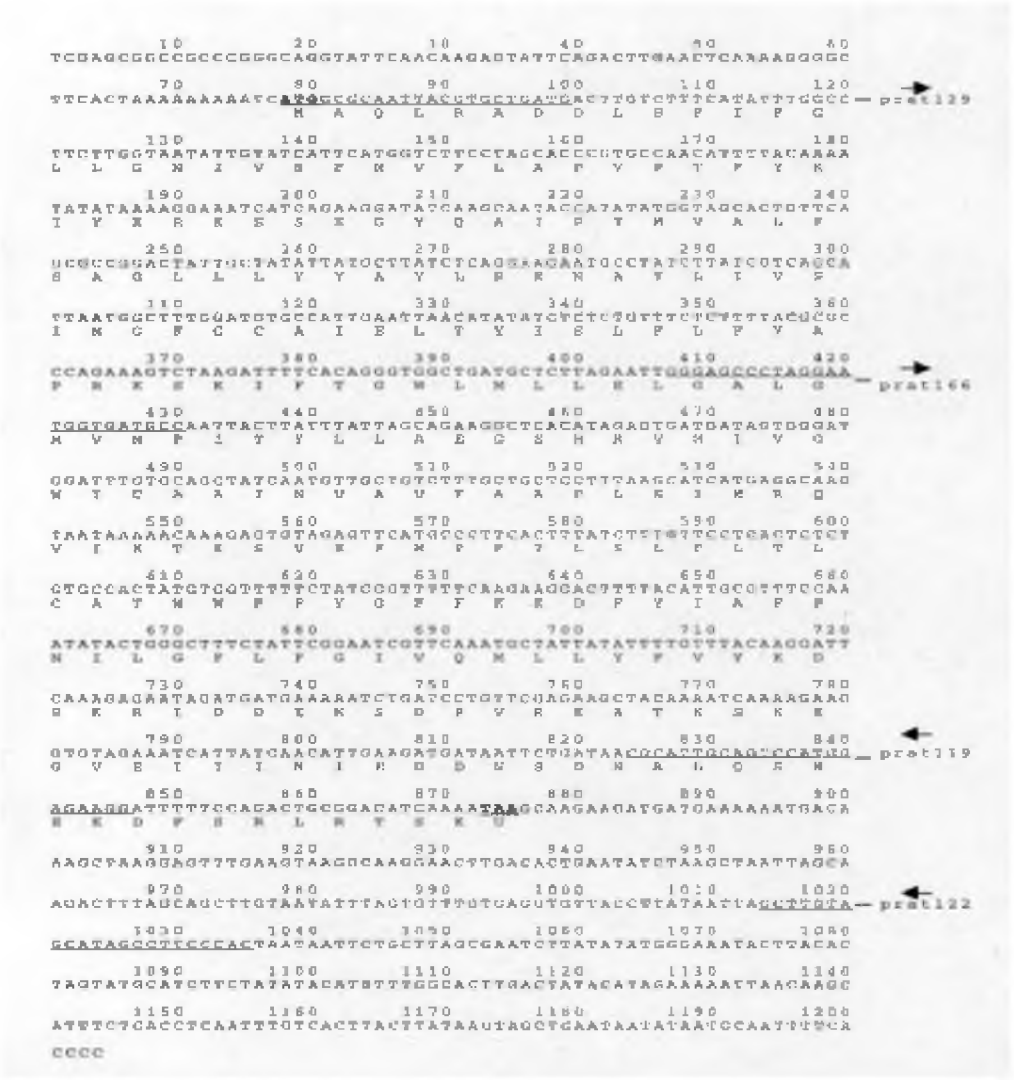
The spatial distribution of *NEC1* mRNA within the nectary tissue was investigated by *in situ* hybridization (Figure 2C). A *NEC1* antisense RNA probe was hybridized *in situ* with longitudinal sections of a flower at stage 2. High levels of *NEC1* transcripts were predominantly observed in the outer nectary parenchyma cells. Low hybridization signals were observed at the base of the petals, near to the vascular tissue. The sense RNA probe gave no signal above the background (data not shown).

Figure 1. cDNA sequence and the putative protein structure of *Petunia hybrida NEC1* gene.

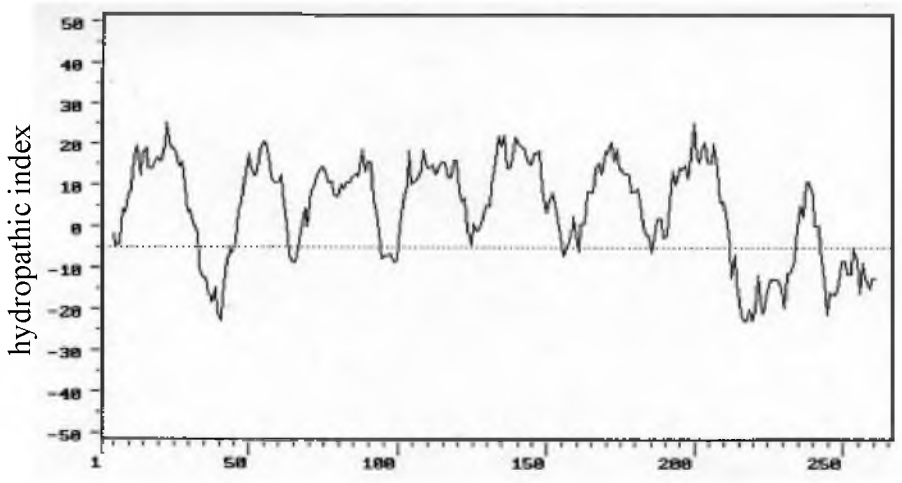
(A) Nucleotide and derived amino acid sequence of NEC1. The translation and stop codons are indicated bold and underlined. *NEC1* gene specific primers Prat 129, Prat 166, Prat 119 and Prat 122 are underlined and indicated beside the figure.

(B) Hydropathy plot of the deduced NEC1 polypeptide. The hydropathic index (Kyte and Doolittle, 1982) is plotted against the amino acid number at an interval of 9 amino acids. The areas above and below the mean index value (- 5) are defined as hydrophobic and hydrophilic, respectively.

A



B


hydropathic index
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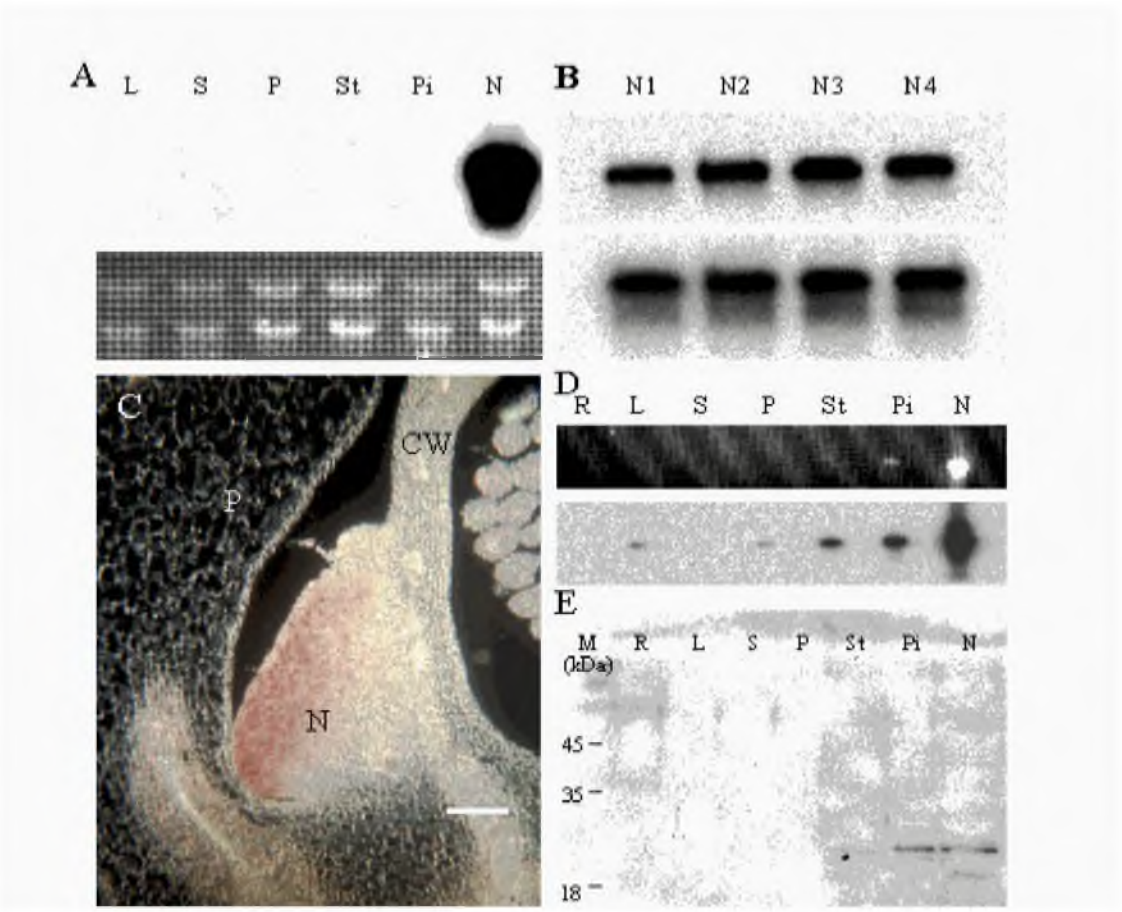

Figure 2. *NEC1* expression analyses in wild-type *Petunia* (W115).

(A) Equal amounts (10μg) of total RNA from leaves (L), sepals (S), petals (P), stamens (St), pistils (Pi) and nectaries (N) were probed with a [^{32}P]-labeled 3' *NEC1* gene specific cDNA fragment of 470 bp; RNA gel was verified by Ethidium bromide (EtBr) staining before blotting (bottom).

(B) Gel blot analysis of total RNA isolated from nectaries of flower stages 1-4 (N1 to N4). Filters were hybridized with full-length [^{32}P]-labelled *NEC1* cDNA (top), and a ribosomal DNA probe (28S rRNA) as a standard (bottom).

(C) Localization of *NEC1* mRNA in nectaries. Longitudinal sections of stage 2 flowers were hybridized to antisense digoxigenin-labelled RNA probes from *NEC1*. Sections were viewed using dark-field microscopy, the signal color being red. Bar = 50 μm.
CW, carpel wall; N, nectary; O, ovules; P, petal.

(D) Total RNA isolated from roots (R), leaves (L), sepals (S), petals (P), stamens (St), pistils (Pi) and nectaries (N) was used for RT-PCR reactions using gene specific primers of *NEC1*: Prat 166 and Prat 122 (Figure 1) to amplify a 600 bp fragment of *NEC1* (top). Gel blot analysis was carried out on PCR products, using the full-length [^{32}P]-labelled *NEC1* cDNA as a probe for hybridization (bottom).

(E) Equal amounts of proteins (20μl) isolated from roots (R), leaves (L), sepals (S), petals (P), stamens (St), pistils (Pi), and nectaries (N) were used for the Western blotting. The blot was probed with NEC1 antibody (1:7000 dilution) and goat anti-rabbit (dilution 1:50000) before detection. M: Marker.

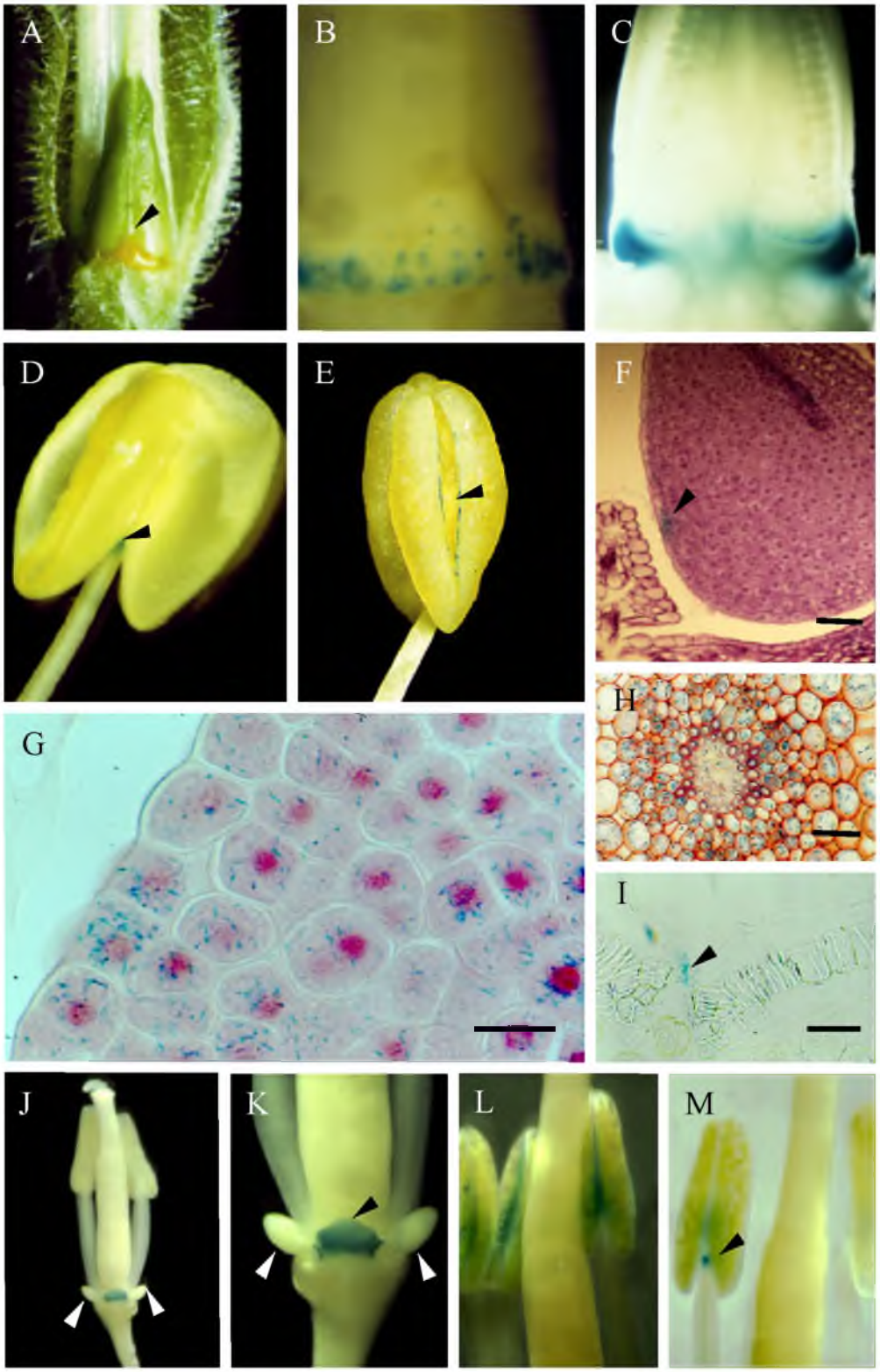
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Figure 3. *NEC1* promoter driven GUS activity in *Petunia hybrida* and *Brassica napus* flowers

(A) Wild type *Petunia* flower (stage 3) with nectary (arrow) at the base of the pistil.

(B) GUS activity in young nectaries of 2 cm flower buds.

(C) GUS activity in nectaries of stage 3 and 4 flowers.

(D, E) GUS activity in stamen of stage 3 flowers, showing GUS staining in the top of the filament (arrow) **(D)** and the anther stomium (arrow) **(E)** just at anthesis.

(F, G) Longitudinal sections through nectaries showing GUS expression in the outer cell layers (arrow) of the nectary in stage 1 flowers **(F)** and in nectary parenchyma cells of stage 4 flower **(G)**. Bar = 50 μm **(F)** and bar = 10 μm **(G)**.

(H) Cross section through region of filament expressing GUS. Bar = 10 μm.

(I) Cross section through region of stomium expressing GUS (arrow). Bar = 10 μm.

(J-M) GUS activity in *Brassica napus* flower **(J)**, specific in lateral nectaries (black arrow), but not in median nectaries (white arrows) **(K)**, it is in the anther stomium **(L)** and the top of the filament (arrow) **(M)**.

A peptide antibody was raised against the last 14 amino acids of the hydrophilic C-terminal part of the NEC1 protein. Immunoblot analysis showed that the peptide antibody hybridizes to a 27 kDa fragment in nectaries, stamens and pistils. The size of this fragment is consistent with that of the putative NEC1 protein (Figure 2E). NEC1 protein is mainly present in nectaries and weakly in stamens and pistils. However, cross hybridization with a larger protein fragment was observed in nectaries, stamens and roots, while some smaller, weakly hybridizing fragments are present in nectaries, pistils and stamens.

NEC1 promoter is active in nectaries during flower development

To study the regulation of *NEC1* expression during flower development, we cloned a *NEC1* promoter fragment of 2140 bp by genome walking. A chimeric gene construct was made that consisted of the *NEC1* promoter fragment fused upstream to the GUS reporter gene. This chimeric gene construct was transferred to *Petunia* line W115 using *Agrobacterium*-mediated transformation. Transgenic plants were tested for GUS expression.

All GUS- positive plants (5 of 20 transformed plants) showed similar GUS activity patterns, though there was variation in the level and the timing of GUS expression among different transformed lines. One plant (T95015), which showed a relatively high level of GUS expression in nectaries, was chosen for further study.

To determine the timing of *NEC1* expression in the nectaries, flowers of different developmental stages of plant T95015 were tested for GUS expression. GUS expression was detectable in nectaries of flower buds from 1.7cm (before

stage 1 flower) and increased during further development of the flower. GUS staining in 1.7cm-long flower buds was visible as blue spots on the surface of the nectary (Figure 3B). Histological examination of longitudinal sections through the nectaries of these flowers revealed that GUS expression was mainly present in the epidermis cells (Figure 3F). GUS expression was highest in nectaries of open flowers, just before and after anthesis (Figure 3C). At these stages, GUS activity was present throughout all the nectary parenchyma cells (Figure 3G).

Plant T95015 was further tested for GUS activity in other floral tissues, leaves and roots. Flower buds of stages 3 and 4 showed GUS expression in specific regions of the stamens, namely the upper part of the anther filament (Figure 3D) and the anther stomium (Figure 3E). Very often only one or two of the anthers in the same flower showed GUS expression. GUS expression in the anther filament was the highest in cells surrounding the central vascular bundle (Figure 3H). Cross sections through the anther revealed that GUS expression was precisely restricted to the stomium cells, which become disrupted during anthesis (Figure 3I).

Occasionally, GUS expression was also observed in the upper region of the ovary and the transmitting tissue of the style (data not shown). No GUS expression was observed in vegetative plant parts, such as leaves, roots and stems.

Brassica napus plants were transformed with the same construct. *Brassica napus* flowers contain one pair of median and one pair of lateral nectaries (Figure 3J, K). The lateral nectaries actively secrete nectar, whereas in median nectaries nectar secretion is very limited. In Brassica, the same expression pattern as in *Petunia* was observed (Figure 3J), showing strong GUS expression exclusively in the lateral nectaries (Figure 3K), the anther stomium (Figure 3L) and the upper part of the anther filament (Figure 3M).

Localization of NEC1 protein

To determine the cellular localization of NEC1 protein, immunolocalization was carried out on longitudinal sections of flowers. The presence of NEC1 protein was visualized by red fluorescence, while counter staining with the DNA-specific dye DAPI was used to visualize the nuclei (Figure 4A, E).

Immunolabeling of longitudinal sections through ovules, petals and nectaries from stage 2 flowers showed that NEC1 protein was present in all nectary cells, the outer parenchyma cells showing the highest concentration (Figure 4C, D, F). Control sections treated with preimmune serum, only showed weak non-specific labeling in the epidermis cells of the nectaries (Figure 4B).

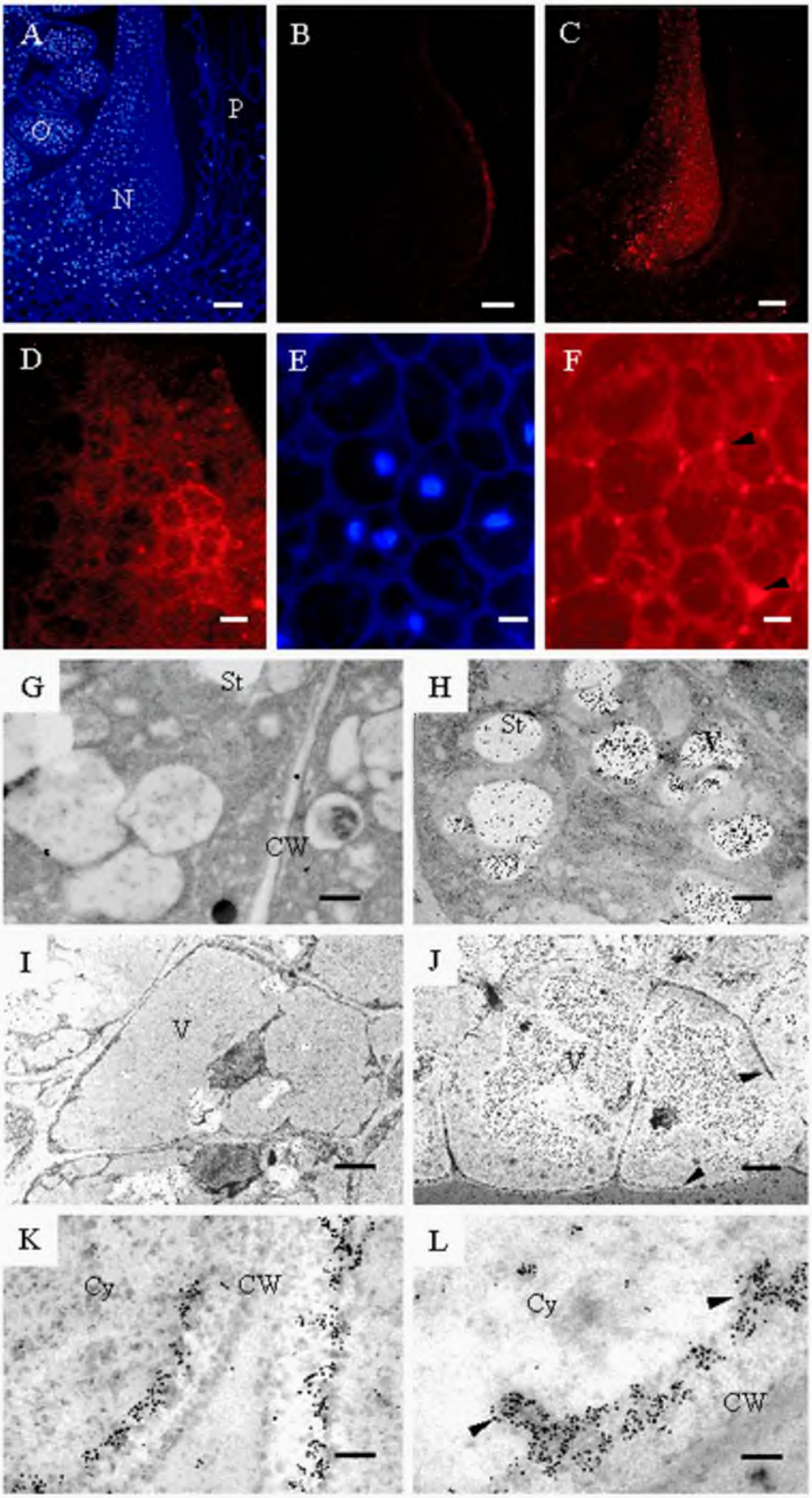
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Figure 4. Immunolocalization of the NEC1 protein in nectaries of *Petunia* flowers.
A-F Immunolocalization in nectaries of stage 2 flowers using fluorescence microscopy;
G-L Immunolocalization using transmission electron microscopy (TEM).
(A) Longitudinal section through ovules (O), petal (P) and nectary (N), showing blue fluorescence of the DAPI stained nuclei. Bar = 50 μm.
(B) Control labeling with pre-immune serum on a similar section through ovules, petal and nectary, showing non-specific labeling in the epidermis of the nectary. Bar = 50 μm.
(C, D) Same section as **(A)**, showing localization of NEC1protein in nectary (red fluorescence). Bar = 50 μm **(C)** and Bar = 5 μm **(D)**.
(E) Nuclei of nectary parenchyma cells stained with DAPI. Bar = 5 μm.
(F) Same section as **(E)**, showing localization of NEC1 in the apoplastic space between parenchyma cells (arrow). Bar = 5 μm.
(G) TEM Control labeling with pre-immune serum, showing little labeling in the nectary cells of stage 2 flowers. Bar = 300 nm.
(H) Localization of NEC1 protein on starch granules and vacuoles in stage 2 flower before anthesis. Bar = 300 nm.
(I) TEM Control labeling with pre-immune serum, showing little labeling in the nectary cells of stage 4 flowers, at this stage cells are full with large vacuoles. Bar = 1 μm.
(J) Localization of NEC1 protein in the region of the plasmalemma (arrow) and vacuoles in outer layer nectary cells of *Petunia*. Bar = 1.5 μm.
(K, L) The NEC1 protein surrounds the plasmalemma along the nectary cell wall **(K)**. Fusion of the vesicles with the plasmalemma followed by excretion (arrow) **(L)**. Bar = 100nm.
St, starch granule; CW, cell wall; V, vacuoles; Cy, cytoplast.

To obtain more specific information, the sub-cellular localization of the NEC1 protein was studied using immunogold labeling and electron microscopy. In nectary sections of stage 2 flowers abundant labeling was observed in small vacuoles and on starch granules (Figure 4H). Control sections that were treated with pre-immune serum showed very little labeling (Figure 4G). In nectary cells of stage 4 flowers large vacuoles were observed, showing very dense labeling (Figure 4J), while minor background labeling was observed (Figure 4I). Specific labeling was present around the plasmalemma (Figure 4J, K) and in small vesicles that appeared to fuse with the plasmalemma (Figure 4L).

NEC1 gene expression and the degradation of starch

The accumulation and hydrolysis of starch in nectaries was studied in relation to *NEC1* expression in a transgenic plant T95015 that expressed GUS under the control of the *NEC1* promoter. Starch accumulated during the development of nectaries and starch concentration was highest in stage 2 flowers. GUS expression at this stage was mainly observed in the epidermal cells (Figure 5A). Prior to anthesis, starch hydrolysis was initiated, which progressed from the outer to the inner nectary cells (Figure 5B). The pattern of GUS expression appeared to follow the temporal events of starch hydrolysis, GUS expression being high in tissues where starch disappeared. After anthesis (stage 4), the majority of starch was hydrolyzed (Figure 5C), while high GUS expression was observed in all nectary cells (Figure 3G, 5C). A similar pattern was observed in the tip of the filament. Starch accumulated before anthesis and GUS expression was low during this stage, being localized in the central vascular bundle (Figure 5D). During anthesis, starch in the filament tip degraded, while GUS expression was the highest in tissues that previously contained starch (Figure 5E).

Ectopic expression of *NEC1*

To investigate the effect of ectopic expression of *NEC1* in *Petunia*, we fused the *NEC1* cDNA to the double enhanced *CaMV 35S* promoter (Figure 6A), introduced the construct into *Petunia hybrida* line W115 and generated 50 independent transgenic plants. Four of 30 transformants showed ectopic expression of *NEC1* RNA in leaves as assessed by Northern blot analysis (Figure 6B). One of the four plants, T91034 was chosen to study the phenotype in a segregating population.
In the 30 progeny plants of the inbred line of T91034, there was a clear segregation of wild type plants, plants with intermediate and plants with a severe phenotype (Figure 6C), in approximately a ratio of 1: 2: 1. Plants that showed a severe phenotype had upwardly curled leaves with a brown tip (Figure 6E, F). In addition, flowering time was slightly delayed compared to wild type plants (Figure 6C). Ectopic expression of *NEC1* RNA was observed in leaves of plants that showed a severe or intermediate phenotype (data not shown). Histological examination of cross-sections through leaves showed that the mid veins of over-expression plants contained on average 3-4 times more phloem bundles compared to wild type plants (Figure 6G, H). The same phenomenon was observed in the minor veins of over-expression leaves (data not shown). Furthermore, in leaves of over-expression plants, palisade parenchyma cells were hardly distinguishable, instead only spongy parenchyma cells were observed (Figure 6I, J).

Discussion

Involvement of *NEC1* in nectar secretion

Nectaries are sink organs that accumulate and secrete nectar that contains considerable quantities of fructose and glucose, whereas in the phloem exudate, sucrose is the only sugar. As discussed by Copeland (1990), the enzyme invertase and sucrose synthase are responsible for the conversion of sucrose to hexoses. However, sugars are also synthesized from the hydrolysis of starch in the nectaries. In *Petunia* flowers, starch accumulation starts already in immature nectaries, and is highest in closed flower buds that just initiate the secretion of nectar. Afterwards, gradual starch hydrolysis takes place, starting in the outer cells of the nectary. During anthesis, a rapid conversion of all the remaining starch takes place, releasing a high amount of soluble sugars into the secreted nectar.

GUS expression under the control of the *NEC1* promoter in *Petunia* nectaries occurred in cells where starch hydrolysis took place. GUS expression is highest in nectaries of flowers after anthesis in which active secretion of nectar takes place and starch hydrolysis was almost complete.

Various suggestions have been made regarding the possible ways of sugar transport into the secretory cells and of nectar elimination from those cells (Fahn, 1979), one of these being the secretion of nectar via vesicles, whose membranes fuse with the plasmalemma. In this respect the observed presence of NEC1 protein on starch grains and vesicles in nectary parenchyma cells suggests a role of *NEC1* in nectar secretion. However, as immunoblot labeling showed some cross-hybridization with other proteins, non-specific labeling cannot be excluded.

Interestingly, the results on transgenic *Brassica napus* plants support our hypothesis that *NEC1* is involved in the process of nectar secretion. Brassica flowers have two pairs of floral nectaries. The lateral pair has an extensive phloem supply and produces most of the floral nectar, whereas the median pair is supplied by limited phloem and produces almost no nectar (Davis *et al.*, 1986 and 1994). *Brassica napus* plants transformed with GUS under the control of the *NEC1* promoter show strong GUS expression exclusively in the lateral nectaries, but no expression in the median nectaries, suggesting the involvement of *NEC1* in nectar secretion.

Since both lateral and median nectaries contain cells exhibiting similar structural features, the disparity in phloem supply between them could be directly responsible for the significant difference in nectar production (Davis *et al.*, 1986). Interestingly, when *NEC1* was ectopically expressed in leaves of *Petunia*, the phloem bundles of the leaves were enlarged compared to the wild type. Over-expression of *NEC1* thus either enhances sugar transport by inducing the development of phloem cells or the enhanced supply of sugars itself induces the development of phloem cells.

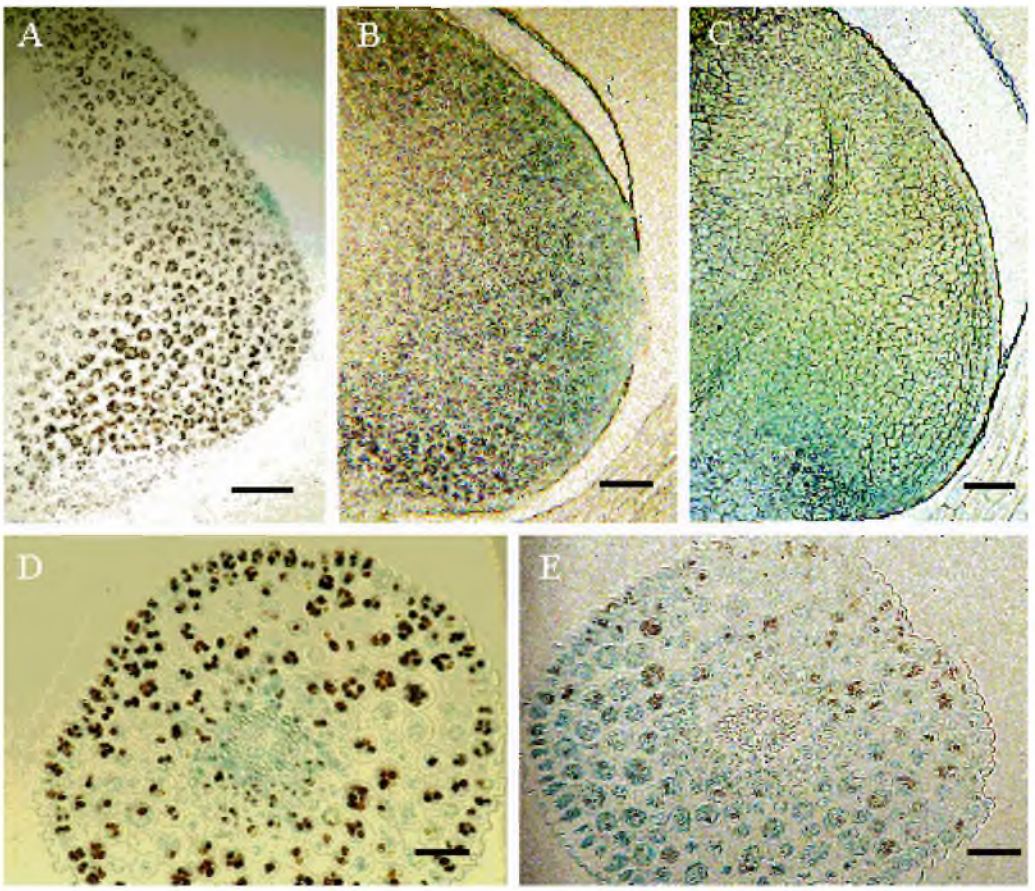

Figure 5. GUS activity under control of the *NEC1* promoter in relation to the presence of starch in nectaries. Starch is stained brown by I_2-KI.

(A, B) Stage 2 flower, before anthesis, showing starch accumulation in all nectary parenchyma cells, while GUS activity is restricted to a few epidermis cells **(A)** or to the outer nectary cells **(B)**. Bar = 50 μm.

(C) Stage 4 flower, after anthesis, starch has been hydrolyzed almost completely, while GUS activity is present in all nectary cells. Bar = 50 μm.

(D) Stage 3 flower, before anthesis, cross-section through the upper region of the anther filament, showing starch accumulation in the outer filament cells. GUS activity is restricted to the central vascular bundle cells. Bar = 30 μm.

(E) Stage 4 flower, after anthesis, cross-section through the upper region of the anther filament, showing high GUS expression in all filament cells, starch has been hydrolyzed almost completely. Bar = 30 μm.

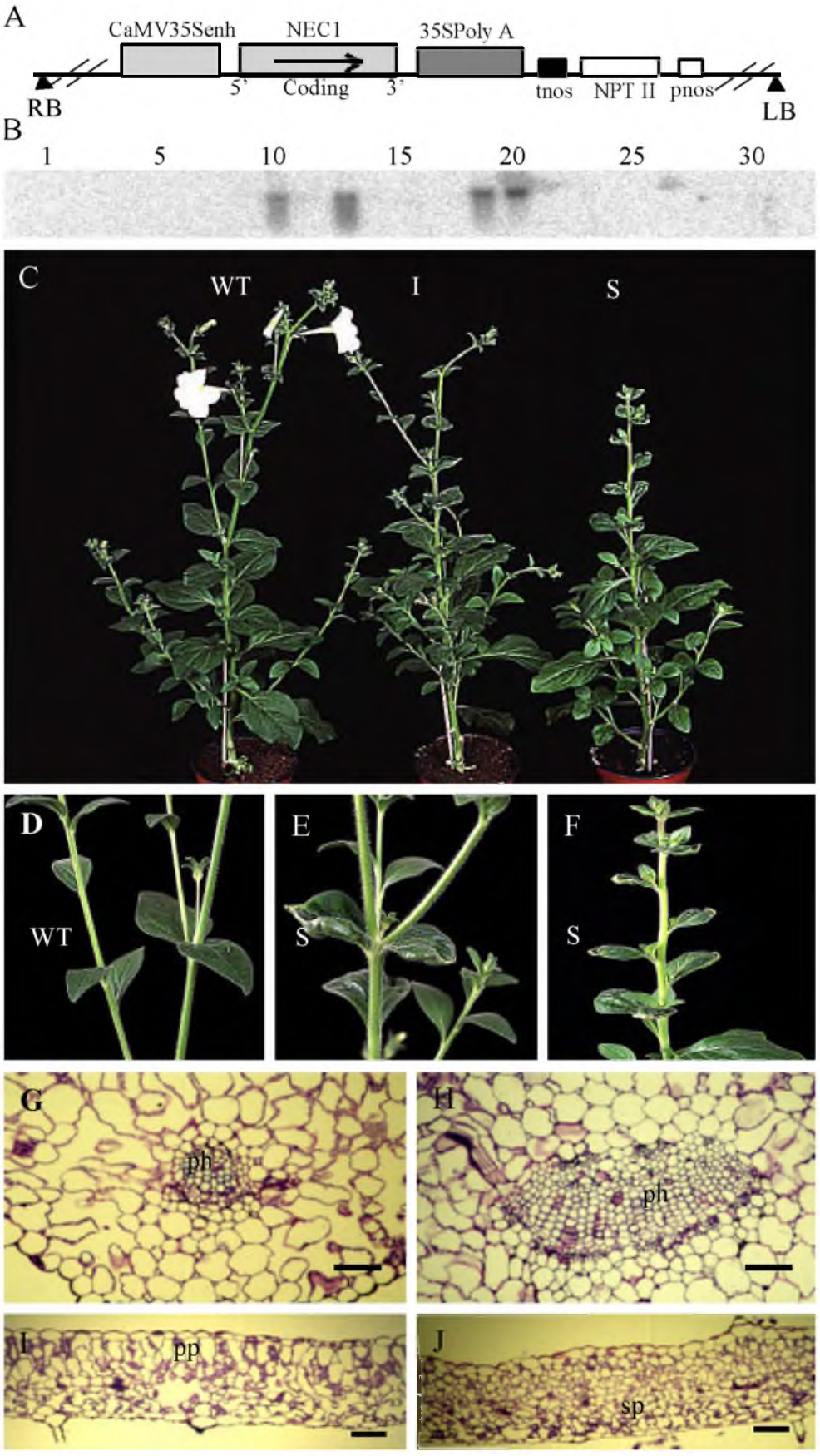
A
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Figure 6. Over-expression of *NEC1* gene in *Petunia hybrida*.

(A) Over-expression construct of *NEC1*. The full-length *NEC1* cDNA was inserted in the sense orientation between the double enhanced *CaMV 35S* promoter and terminator.

(B) *NEC1* RNA ectopic expression in leaves of primary transformants. Filter was hybridized with the full-length [^{32}P]-labelled *NEC1* cDNA. Lane 1-30, 30 independent lines, lane 20: plant T91034.

(C-F) Progeny phenotypes of the plant T91034: wild type plant (WT) **(C, D)**, intermediate plant (I) **(C)** and severe phenotype plant (S), showing upwardly curled leaves with a brown tip **(C, E, F)**.

(G, H) Cross section through region of leaf mid veins of wild type **(G)** and over-expression phenotype plants with more phloem bundle (ph) **(H)**. Bar = 10 μm.

(I, J) Cross section of leaves of the wild type **(I)** and over-expression phenotype plants **(J)**. pp, palisade parenchyma; sp, spongy parenchyma. Bar = 10 μm.

NEC1 expression and anther dehiscence

In *Petunia*, very weak RNA expression of *NEC1* was detected in the stamens. Further analysis of transgenic *Petunia* plants that express GUS under the control of the *NEC1* promoter, showed that GUS activity in the stamens is restricted to some very specific regions. Strong GUS expression was present in the upper region of the filament and in the anther stomium. Remarkably, similar results were obtained with transgenic *Brassica* plants transformed with the same construct. The stamen filament is known to be a tissue through which the anther is supplied with nutrients during pollen development (Clément *et al.*, 1996). It is suggested that during filament elongation, the filament cells use sugars for the elongation. During anther dehiscence of *Gasteria verrucosa*, a sudden disappearance of all starch from the filament was observed (Keijzer *et al.*, 1987). It was suggested that this timed conversion of starch into sugars might cause an osmotic retraction of water from neighboring tissues and promote dehydration of the dehiscing anther. Indeed, Schmid and Alpert (1977) already provided experimental evidences that anther dehiscence is not just a simple desiccatory process. In *Petunia*, GUS expression driven by the *NEC1* promoter in the filament was only detected in flowers just before or after anthesis, the cells with highest GUS expression being located respectively around the central vascular bundle and the filament parenchyma cells. Remarkably, the strongest GUS expression was again observed in cells, where starch degradation had already taken place. Although we have no experimental evidence that *NEC1*-driven GUS activity and starch degradation are linked, the concomitant occurrence of both phenomena in different tissues and developmental processes is challenging. If *NEC1* is involved in sugar

transport or metabolism, its possible role in anther dehiscence could be the release of sugars from starch to build up the osmotic pressure needed to withdraw water from the anthers.

Similar to the filament, GUS expression under the control of the *NEC1* promoter could only be detected in the anther stomium of flowers just before or after anthesis. The anther stomium is ruptured during anther dehiscence by swelling of the epidermis and the endothecium (Keijzer and Willemse, 1988). It is presumed that breakdown of starch from the epidermis and endothecium causes an increase in the osmotic pressure and subsequent swelling of these cells. However, GUS expression was only detected in the stomium cells and not in the epidermal or endothecium cells. The role of *NEC1* in this process is therefore not clear.

The results obtained in the present study are an important step towards the elucidation of the molecular regulation of nectary development and nectar secretion. Based on the specific locations of *NEC1* expression and the function of these tissues, it is assumed that *NEC1* may function as a regulator in sugar transport or sugar metabolism during the process of nectar secretion and anther dehiscence. Further research on the effects of down-regulation of *NEC1* gene will further elucidate the function of the gene in nectary development, nectar secretion and anther dehiscence.

Experimental procedures

Plant materials

Petunia hybrida plants of Wild-type W115, *Brassica napus* plants of Topas and the transgenic plants derived from *Petunia* and *Brassica* were grown under normal greenhouse conditions.

Cloning of *NEC1* by Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR)

A nectary-specific cDNA clone was isolated by using the mRNA Differential Display system (Genhunter Corporation, Nashville, TN 3721, USA). Total RNA was isolated from nectaries, sepals, pistils, stamens and young leaves of *Petunia hybrida* according to Verwoerd *et al.* (1989). RT-PCR was carried out following the protocol of Genhunter Kit. The nectary-specific bands were cut out from the gel and the DNA was purified and reamplified according to the manual. The fragments were cloned into a PMOSBlue T-vector (RPN 1719, Amersham Little Chalfont UK) for Northern blot analyses and sequencing. After electrophoresis of the PCR product, a 470 bp fragment was obtained that showed a very high expression in nectaries.

To generate the full-length cDNA of this fragment, 5' RACE- PCR was carried out, using the Marathon ^{TM} cDNA Amplification Kit of Clontech (catalog K1802-1). The gene-specific primer Prat 122 (5'-GTGGGAAGGCTATGCTACAAGC-3') and nested gene-specific primer Prat 119 (5'-CCTTCTCCATGGACTGCAATGCG-3') (Figure1) were used for PCR reactions together with the adapter primers from the kit. Afterwards, a fragment of ~ 850 bp was extracted from the gel, purified and cloned into a PMOSBlue T-vector for sequencing.

After combining (overlapping) the sequences of the separate 3' and 5' cDNA clones, the full length cDNA was cloned by PCR, using the gene specific primers Prat 129 (5'-ATGGCGCAATTACGTGCTGATG-3', Figure1) and Prat 122.

DNA sequencing and sequence analysis

DNA sequences were determined by the dideoxynucleotide chain termination method (ABI PRISM^{TM} Ready Reaction DyeDeoxy^{TM} Terminator Cycle Sequencing Kit, P/N 402078, Perkin Elmer).

The protein structure was predicted using the CAOS/CAMM program (Protein analysis 1991, Genetics Computer Group inc., Medison, Wisconsin, USA).
The *NEC1* accession number is Banklt362739 AF313914.

RNA gel blot analysis

Approximately 10µg of glyoxal (1.5 M) denatured total RNA (was separated by electrophoresis on a 1% agarose gel, containing ethidium bromide to verify the equal loading of the different RNA samples. Gels were blotted overnight to Hybond N^{+} membranes (Amersham) in 25mM phosphate buffer (pH 6.5). Membranes were hybridized under standard conditions at 65 °C, with [^{32}-P]-labelled *NEC1* cDNA probes as described by Angenent *et al.* (1992). Normally, the hybridized blot was exposed one day to X-ray film.

***In Situ* RNA hybridization**

Flower buds of *Petunia* W115 were fixed and embedded in paraffin. A standard protocol for in situ hybridization was used, as described by Cañas *et al.* (1994). Shortly, a digoxigenin-labeled RNA probe of *NEC1* was synthesized by in vitro transcription using the pSPT18/pSPT19 vectors (Boehringer Mannheim). For the synthesis of antisense RNA, a PCR fragment corresponding to the nucleotides 79 to 1036 was introduced into pSPT19. In vitro antisense RNA transcripts were made using T7 RNA polymerase. Transcripts were partly hydrolyzed by incubation at 60°C in 0.1M Na_2CO_3 buffer, pH 10.2, for 50 min. Hybridization and immunological detection were performed as described by Canãs *et al.* (1994).

Sections were viewed on a Nikon OPTIPHOT microscope and photographed under dark field conditions with a Nikon HFX-II camera.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

DNase treatments were carried out on total RNA from roots, leaves, sepals, petals, stamens, pistils and nectaries, using the RNA MessageClean^{TM} Kit (Genhunter Corporation Brookline USA, cat. No. M601). 0.2 µg RNA of each sample was used for reverse transcription (RT), using the oligo-dT primer T12MG from the Genhunter Kit. 1.0 µl of each RT reaction was used as template for PCR amplification with *NEC1* gene specific primers Prat 166 (5'-GGGAGCCCTAGGAATGGTGATGCC-3', Figure 1) and Prat 122 (Figure 1). Amplification involved 1 cycle with a denaturation time of 2 min at 94°C, followed by 30 cycles with a denaturation time of 0.5 min at 94°C, annealing time of 1 min at 52 °C and an extension time of 2 min at 72°C. PCR products were subjected to electrophoresis on a 1% agarose gel (Figure 3D). The gel was blotted overnight to Hybond N^+ membrane (Amersham) under alkaline conditions and hybridization was carried out under standard conditions at 65 °C, using full-length [^{32}-P]-labelled *NEC1* cDNA as a probe.

Isolation of *NEC1* promoter by genome walking

The promoter fragment of *NEC1* was cloned using the genome walker protocol (PT3042-1) and kit as provided by Clontech Laboraties. Briefly, genomic DNA from *Petunia hybrida* was digested with 5 different blunt cutting restriction enzymes. GenomeWalker adapters were ligated and PCR reactions were carried out on each GenomeWalker "library" with a gene specific, reversed primer Prat 148 (5'-CCAAG-AAGGCCAAATATGAAAGAC-3', comprising the nucleotides 105 to 128 of the *NEC1* cDNA) and the adapter primer from the kit (AP1). PCR products were subjected to a second round of PCR, using the nested adapter primer AP2 and the nested gene specific, reversed primer Prat 149 (5'-AAGTCATCAGCACGTAATTGCGCC-3', comprising nucleotides 81 to 104 of the *NEC1* cDNA). From the second PCR a ±2kb fragment was isolated from the *Stu*I library, which was cloned in the PMOSBlue T-vector for sequencing. A 2140 bp DNA fragment of the *NEC1* promoter was obtained, including the translation start of *NEC1* cDNA.

Constructions of binary vector and plant transformation

The GUS gene was cloned downstream of the *NEC1* promoter. Briefly, the *NEC1* promoter fragment was amplified by PCR and cloned into a pBluescript-derived

vector containing the GUS reporter gene and nos terminator, resulting in the *NEC1*-promoter/GUS translational fusion.

NEC1 over-expression construct: the full-length cDNA of *NEC1* in sense orientation was cloned downstream of the double enhanced *CaMV 35S* promoter (Figure 6A).

The *NEC1* promoter/*GUS*/nos and the *CaMV 35S* promoter/*NEC1*/ nos fragments were ligated into a pBIN-derived vector pBINPLUS (Van Engelen *et al.*, 1995).

The recombinant vectors were transferred via *Agrobacterium tumefaciens* (LBA4404) to *Petunia* variety W115, using the standard leaf disk transformation method (Horsch *et al.*, 1985). After shoot and root induction on kanamycin (250µg/ml) selection media, plants were transferred to soil in the greenhouse. The transgenic plants were verified by DNA gel blot analysis.

GUS-reporter construct was transferred via *Agrobacterium tumefaciens* (LBA4404) to *Brassica napus* cv. Topas, using microspore-derived embryos as targets for transformation (Custers, unpublished).

Histochemical β-Glucuronidase (GUS) activity assay

Different plant parts of kanamycin-resistant plants were analyzed for the distribution of ß-glucuronidase activity (GUS) using the method described by (Jefferson *et al.*, 1987).

Microscopy

For light microscopic analysis, plant material was fixed, sectioned and stained according to Colombo *et al.* (1997b).

Starch staining

Sections were stained for the presence of starch, using I_2-KI solution, containing 0.3 g I_2, 1.0 g KI, in 100ml water.

Immunoblot analysis and in situ immunolocalization

A peptide antibody was raised in rabbits against the last 14, C-terminal amino acids of the predicted protein of NEC1 by Eurogentech (Belgium). The peptide sequence is (H_2N -QSMEKDFSRLRTSK- COOH). Two rabbits were immunized and three boost injections were given (boost 1in 16/03/98, boost 2 in 30/03/98 and boost 3 in 27/04/98). One month after the last boost injection, the serum was used for testing. Pre-immune serum was taken before injection and used as control.

Total protein fraction was isolated from roots, pistils, stamens, petals, sepals, leaves and nectaries of *Petunia hybrida*, according to the method described by Angenent *et al.* (1992). Equal amounts of proteins were loaded on a 12% SDS-page gel. After electrophoresis the proteins were transferred to a nitrocellulose membrane by Western blotting. The blot was hybridized with the NEC1 antibody (dilution 1:7000). The secondary antibody was a goat anti-rabbit/HRP (dilution 1:50000) conjugate. Detection was carried out using the ECL Plus™ Western Blotting detection reagents (Amersham Pharmacia Biotech). The blot was then exposed to Fuji film for 30min.

For *in situ* immunolocalization, the sample fixations, embedding and immunolabeling procedures for light microscopy, were as described by Wittich *et al.* (1999). Immunolocalization was performed using a Nikon Labophot fluorescent microscope.

For immunocytochemistry on ultrastructural level, sections through nectaries were fixed in 4% paraformaldehyde buffered with PBS, pH 7. After rinsing, the samples were dehydrated through a graded series of ethanol, and embedded in London Resin White (LR White). Ultrathin sections were cut with an LKB Bromma 2088 ultratome, and the sections were collected on formvar-coated nickel grids. The grids were incubated with NEC1 antibody and its corresponding pre-immune serum, both diluted 1:100 in 1% BSAc (acetylated Bovine serum albumin) in PBS buffer, for 2 hours. Then, the grids were incubated for 1.5 hour with goat anti-rabbit IgG conjugated with 0.8 nm gold particles (AURION), diluted 1:30 in BSAc buffer. After silver enhancement with AURION R-GENT SE-FM Kit, sections were stained with uranyl acetate. Sections were observed with a JEOL JEM-1200 EXII transmission electron microscope.

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CHAPTER 4

PARTIAL GENE SILENCING OF *NEC1* RESULTS IN EARLY OPENING OF ANTHERS IN *PETUNIA HYBRIDA*

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Summary

The *NEC1* gene isolated from *Petunia hybrida* is highly expressed in nectaries, and very localized in stamens, particularly in the anther stomium cells and the upper part of the filament. To elucidate the function of the *NEC1* gene, co-suppression was performed for down-regulation of *NEC1* expression, and transposon insertion mutagenesis was used to knock out the *NEC1* function. Among the transgenic plants and the plants with *dTph1* inserted in the *NEC1* gene, plants showed an "early open anther" phenotype. In this mutant phenotype, the anthers already open in young flower buds (1.8 cm) that still contain immature pollen, resulting in poor pollen quality and impaired pollen release. The results obtained reveal that *NEC1* might be involved in the development of stomium cells, which are ruptured during the normal process of anther dehiscence to release mature pollen. Southern blot analysis indicated the presence of a highly homologous *NEC1*-like gene, namely *NEC2*, in the *Petunia hybrida* genome. The presence of *NEC2* was confirmed by segregation analysis and sequencing of genomic clones. The implications of these results and the possible reasons that no visual recognizable phenotype in nectaries could be produced by co-suppression or transposon insertion mutagenesis are discussed.

Keywords: nectary, stomium, gene silencing, anther dehiscence, *Petunia hybrida*.

Introduction

Floral nectaries secrete nectar, a sugar-containing fluid that attracts pollinating insects. The molecular regulation of nectary development and nectar secretion is poorly understood. Recently, we cloned a new gene, *NEC1* that appears to be involved in the process of nectar secretion (Ge *et al.* 2000). However, the exact function of *NEC1* has not yet been elucidated.

The *NEC1* gene is highly expressed in nectaries of *Petunia hybrida* and weakly in stamens. In stamens, the expression was confined to the anther stomium and the upper part of the anther filament (Ge *et al.* 2000). To elucidate the biological function of the *NEC1* gene, we studied the effects on nectary and anther phenotypes by down regulation of *NEC1* expression by co-suppression and by loss of function of *NEC1* by transposon insertion mutagenesis. Inhibition of a specific gene by co-suppression is based on the transcription of a transgene causing down regulation of the mRNA level of the homologous endogenous gene. In *Petunia*, co-suppression has been successfully

applied to down regulate genes involved in flower development (Angenent *et al.* 1993, 1995a and 1995b). Inactivation of a gene by insertion of the transposable element *dTph1* is an alternative strategy to establish a loss-of-function phenotype of a specific gene in *Petunia* (Koes *et al.* 1995). The transposon *dTph1* element, which was isolated from the *DfrC* gene of *Petunia hybrida* line W138, can be activated by an activator element *ACT1* (Huits *et al.* 1995). The line W138 contains over 200 copies of *dTph1*, which induces new mutations at a high frequency (Gerats *et al.* 1990; Souer *et al.* 1995; Colombo *et al.* 1997a). An insertion of a *dTph1* transposon in the gene of interest can be identified by a PCR-based screening strategy (Zwaal *et al.* 1993) that has been widely applied in *Petunia hybrida* (Koes *et al.* 1995).

In this chapter, we present the results obtained by co-suppression and transposon insertion mutagenesis of *NEC1* in *Petunia hybrida*.

Results

The *NEC1* gene, is highly expressed in nectaries (Figure 1C) and very localized in the anther stomium (Figure 1 D, E) of *Petunia hybrida* (Ge *et al.* 2000).

Down-regulation of *NEC1* expression results in an "early open anther" phenotype

A co-suppression approach was used to inhibit the expression of the *NEC1* gene in transgenic *Petunia* plants. A binary vector containing the full-length cDNA in sense orientation, downstream of the cauliflower virus *CaMV 35S* promoter (Figure 2A) was introduced into *Petunia hybrida* line W115. In all, fifty independent transgenic *Petunia* plants were generated.

Five of the 50 plants hardly produced selfed seeds, and showed poor germination of pollen in vitro. When transgenic plants T90025, T90026 and T90028 were tested on pollen germination medium of *Petunia*, an average of only 12% pollen germination was observed, compared to about 90% germination in the wild type W115 (Figure 2B-E). To assess down-regulation of *NEC1* expression, Northern blot analysis was performed on primary transformed plants. Plants T90025, T90026 and T90028 showed 50-60% reduction of RNA expression in nectaries (Figure 2F). Histological analysis of nectaries and HPLC analysis of nectar sugar composition revealed no differences between wild type W115 and the transgenic co-suppression lines (data not shown). From plant T90028, a T2 population was obtained after selfing, which was analyzed for segregation of the phenotype. Six out of 30 plants showed segregation for the phenotype that was characterized by "early open anther". Southern blot analysis revealed that these

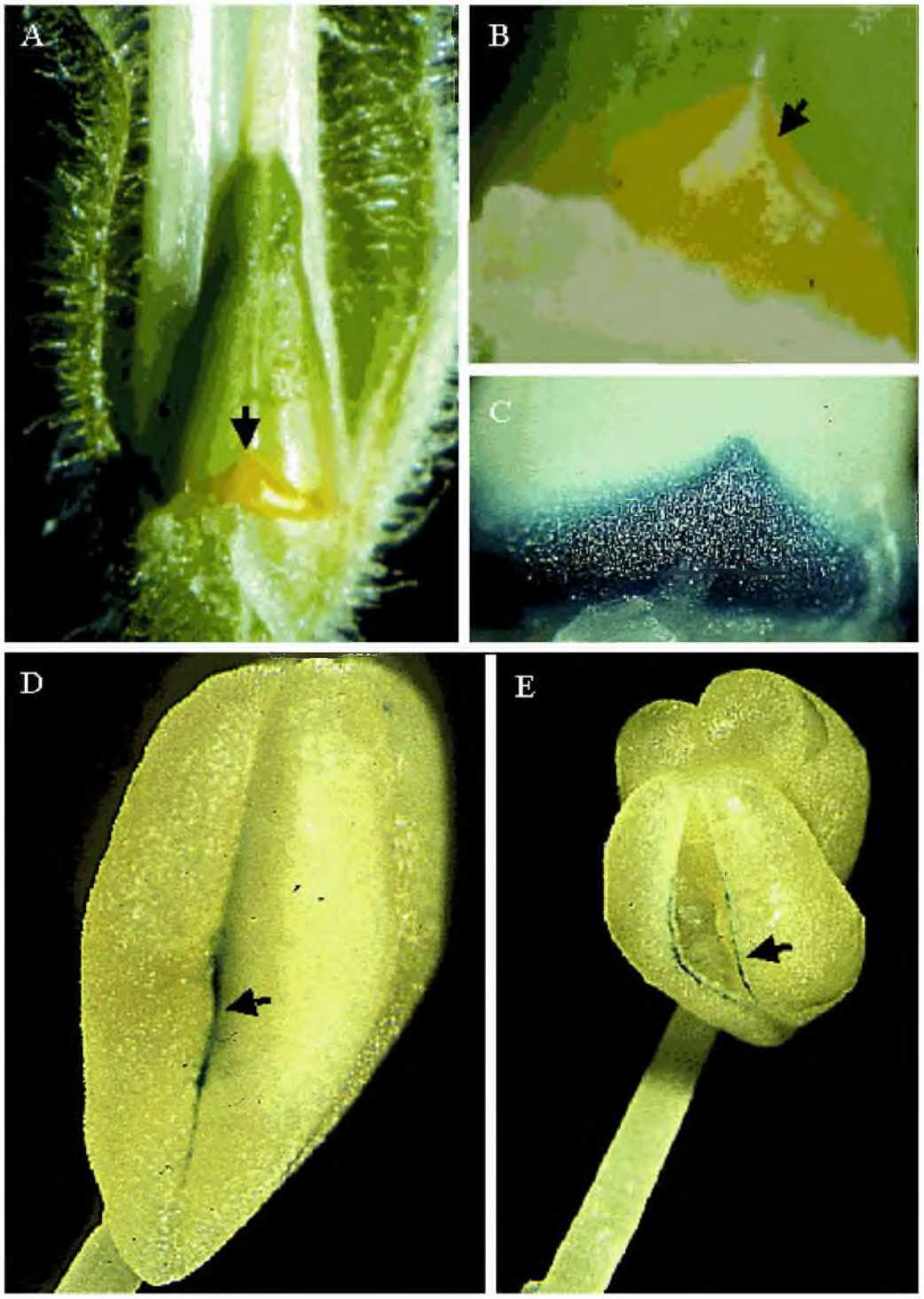


Figure 1. GUS activity under the control of the previously cloned *NEC1* promoter (Ge *et al.* 2000) in *Petunia hybrida* flowers

(A, B) Wild type *Petunia* flowers with nectary (arrow) at the base of the pistil.

(C) GUS activity in nectary.

(D, E) GUS activity in the anther stomium (arrow).

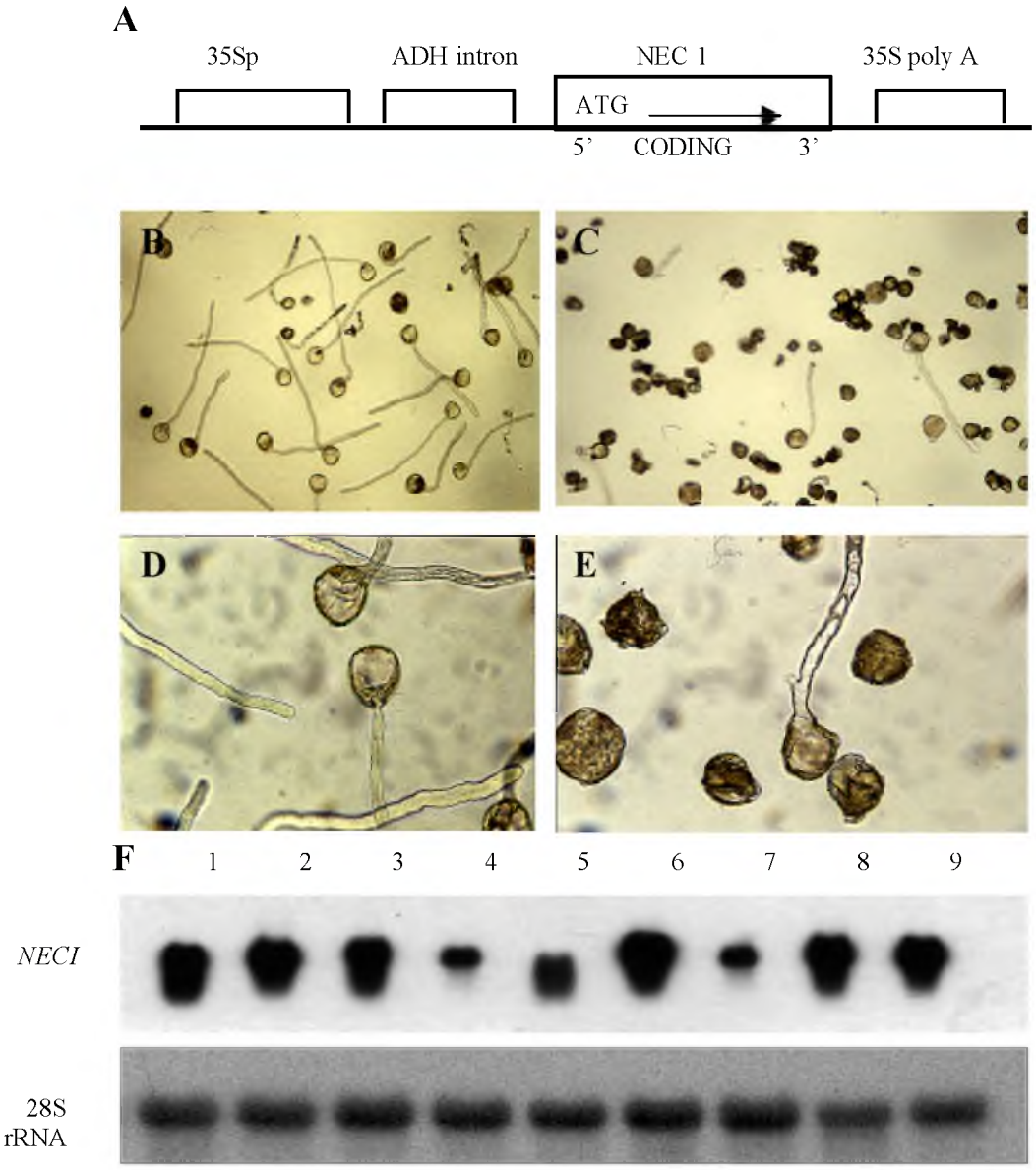


Figure 2. Analysis of down-regulation of *NEC1* by co-suppression in primary transformed *Petunia* plants.

(A) Co-suppression construct, comprising the full-length *NEC1* cDNA in sense orientation under control of the *CaMV 35S* promoter and ADH intron.

(B-E) In vitro germination of pollen grains of wild type **(B, D)** and primary transformed plant T90026 **(C, E)**, 10X **(B, C)** and 50X **(D, E)**.

(F) RNA expression of *NEC1* in nectaries of independent primary transformed plants. Membrane was hybridized with full-length [^{32}P]-labeled *NEC1* cDNA (top), and ribosomal DNA probe (28S rRNA).

Lanes 1-8: Primary transformed plants, T90020, T90022, T90024, T90025, T90026, T90027, T90028 and T90029. Lane 9: wild type (W115).

plants were transgenic (data not shown). The "early open anther" phenotype shows that the anthers are already open in very small (1.8 cm) flower buds (Figure 3A, B), with poor germination of pollen in vivo and in vitro as described above for the primary co-suppression plants. In wild type plants, the anthers open at the stage when the flowers are mature (6 cm).

Histological sections through the anthers of wild type flowers and flowers of co-suppression plants were carried out to examine the process of "early open anther" in more detail. In 2.5 cm buds of wild type flowers the stomium was still intact (Figure 3D), consisting of several vital cells and underlying the stomium two cell layers were visible. At this stage the connectivum had not yet degenerated (Figure 3C). In co-suppression flowers of the same stage, stomium cells were ruptured, while the connectivum had already retreated (Figure 3E). In 6 cm long wild type flowers at anthesis, the stomium cells were degenerating (Figure 3H) and the connectivum was shrinking (Figure 3G). In mature flowers of the co-suppression plants the anthers closed, because the locules bent inwards (Figure 3I). The pollen quality of the co-suppression flowers was poor; in 6 cm flowers almost no vital pollen was observed (Figure 3I). The "early open anther" phenotype was not very severe, it was only observed in 1-3 of the five anthers per flower. In addition, not all the flowers of one plant showed a phenotype.

Because the localized expression of *NEC1* in the stomium results in extremely weak hybridization signals on a Northern blot, the reduction of *NEC1* expression in "early open anthers" was assessed by RT-PCR. *NEC1* expression was reduced in anthers as well as in nectaries (Figure 3J). For the anthers, the location of the phenotype in the anther stomium exactly coincides with the location of GUS expression under the control of the *NEC1* promoter (Figure 1D, E). However, reduced *NEC1* mRNA expression in nectaries did not result in a detectable nectary phenotype.

Screening for plants with a *dTph1* insertion in *NEC1*

To screen for insertion of the transposable element *dTph1* in *NEC1*, a three-dimensional PCR-based screening strategy was used. In total, 2410 plants were screened, using sets of *NEC1* gene-specific primers (GSP) and transposon (*dTph1*) specific primers (TP1 and TP2) (Figure 4A). The A3 DNA pool library represents 960 plants that are three-dimensionally divided over 8 rows (1-8), 10 columns (9-18) and 12 blocks (19-30) (Figure 4B). Amplification of fragments flanking a *NEC1* insertion was detected after hybridization with [^{32}P]-labeled *NEC1* cDNA. Whereas PCR amplification resulted in a similar banding pattern in all the lanes (data not shown), they exhibited differential hybridization patterns (Figure 4B), displaying a hit in plant TT249 (row 1, column 10 and block 22). The selfed seeds of this plant were sown, but because of poor germination, only one plant was obtained.

To verify transposition of the *dTph1* insertion, all the branches were analyzed by PCR. The results revealed that five of the seven branches still contained the insertion (Figure 4C).

The flowers of the positive branches were crossed with the wild type *Petunia* line W115 to stabilize the *dTph1* insertion by eliminating the activator *ACT1*, which encodes a transposase that is responsible for the instability of the transposable element. The analysis of the progeny of these crosses showed that the insertion was heritable and present in about 50% of the F1 plants (Figure 4D). Cloning and sequencing of the fragments flanking the transposon insertion showed that the *dTph1* element was inserted in the coding region at the 3' end of *NEC1* in plants 1, 7, 9, 19 and 23. Plant 3 has two *dTph1* element insertions, of which one is located in an intron and another in the 3' end exon of *NEC1* similar to the other plants. Several plants with only one *dTph1* insertion in the exon region were chosen for further analysis.

DTph1 insertion results in reduced *NEC1* expression and "early open anther" phenotype

In the F2 progeny of W115 × TT249, it was then expected that one out of 16 plants contains a stabilized homozygous transposon insertion in *NEC1*, whereas one out of 4 plants was expected to be homozygous for the *dTph1* insertion in *NEC1*, regardless of the presence of *ACT1* (Figure 5A). Southern analysis of 90 F2 plants revealed that no homozygous insertion mutants could be detected (Figure 5C). The hybridization patterns indicated the presence of either wild type plants without insertion (plants 6, 9, 10, 16, 18 and 24) or plants heterozygous for the insertion.

The lethality of the gametes as a cause, was excluded by the observation that the *dTph1* insertion was transmitted to the progeny plants, when plants with the *dTph1* insertion in *NEC1* were used as male or female for crosses with the wild type W115. Neither could the absence of homozygous insertion plants be explained by embryo lethality, as selfing of "heterozygous" plants (e.g. plants 2, 7, 14 and 19 in Figure 5C) resulted in fruits in which the embryos developed into viable seeds. Embryo lethality could have resulted in a ratio of 1: 4, lethal: viable embryos (data not shown). In addition, germination and plantlet development were normal and precocious death of the plantlets was not observed.

Eliminating the possible lethality of a homozygous insertion mutant, the presence of a *NEC1*-like gene in *Petunia* DNA was considered on the basis of the Southern blot pattern, as explained in Figure 5B. In case of two highly similar genes, the F2 progeny is expected to segregate in a ratio of 1: 2: 1, $A_1A_1A_2A_2$: $A_1a^T{}_1A_2A_2$: $a^T{}_1a^T{}_1A_2A_2$. Indeed, Figure 5C shows that the segregation of the F2 population basically fits with this ratio, indicating the presence of another related gene of *NEC1* in the genome.

As might be expected, the plants that have a homozygous transposon insertion in only one of the two highly similar genes of *NEC1*, if they are both transcribed, can exhibit no phenotype in the nectaries. Remarkably, we observed the occurrence of the "early open anther" phenotype in flowers from plants homozygous for the *dTph1* insertion in one of the two highly similar genes of *NEC1*. The phenotype is identical to the one as described above for down-regulation of *NEC1* expression by co-suppression (Figure 3). However, the "early open anther" phenotype as affected by *dTph1* insertion was more severe, showing on average 3 mutant anthers per flower. The RNA expression levels of *NEC1* in nectaries were compared by selecting plants exhibiting the "early open anther" phenotype ($a^{T}_{1}a^{T}_{1}A_2A_2$), less sever phenotype plants that were heterozygous ($A_1a^{T}_{1}A_2\ A_2$) and the homozygous wild type plants ($A_1A_1A_2A_2$).

Figure 5E shows a reduced level of RNA expression in plants homozygous for the *dTph1* insertion (lanes 4,5) and a less reduced expression level in plants heterozygous for the *dTph1* insertion (lanes 6, 7) when compared to the wild type plants (lanes 1, 2 and 3).

Figure 3. Characterization of the "early open anther" phenotype in T2 population of co-suppression transformant T90028.

(A) The anthers already opened in small flower buds of 1.8cm (right, arrow). In wild type buds (Left) the anthers are still closed.

(B) Closed flower bud (4 cm) with opened anther (arrow), containing immature pollen.

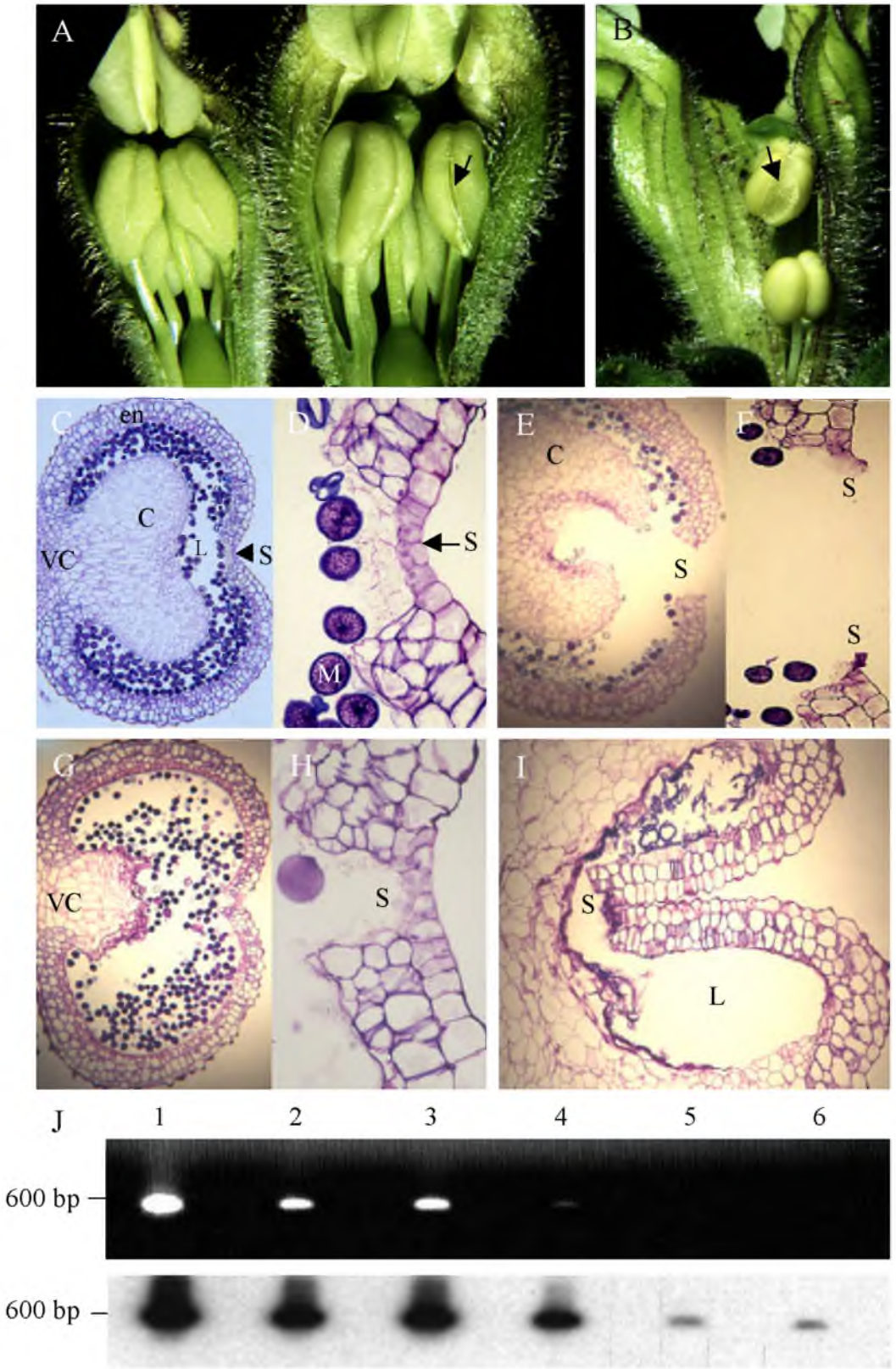
(C-F) Histological examination of cross sections through the anthers of 2.5cm long flower buds. Wild type W115, 50X **(C)** and 400X **(D)**. "Early open anther" phenotype, 50X **(E)** and 400X **(F)**.

(G-I) Histological examination of cross-sections through the anthers of 6cm long flower buds, just at anthesis. Wild type W115, 50X **(G)** and 400X **(H)**. "Early open anther" phenotype, 300X **(I)**.

VC: vascular cylinder; C: connectivum; L: loculus; S: stomium; M: microspore; EN: endothecium.

(J) Gel blot analysis (top) and hybridization (bottom) of RT-PCR on RNA isolated from nectaries and anthers of Wild type W115 plants and "early open anther" phenotype plants.

Lanes 1-3: nectaries from wild type (1) and transgenic lines T90026 (2), T90028 (3), Lanes 4-6 anthers from wild type (4) and transgenic lines T90026 (5), T90028 (6).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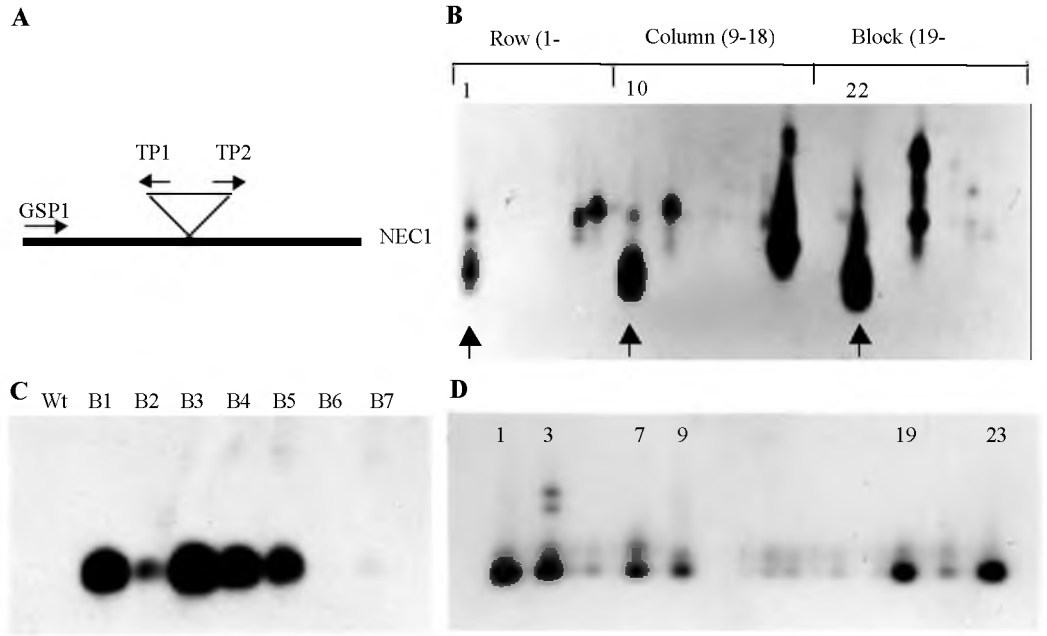


Figure 4. Transposon insertion mutagenesis of *NEC1*.

(A) Schematic representation of primer combinations used for screening: *NEC1* gene specific primer (GSP1, Prat 119) in combination with the two transposon specific primers (TP1 and TP2).

(B) 3D screening of 960 plants divided over 8 rows, 10 columns and 12 blocks. PCR products were analyzed by DNA gel blot analysis and hybridized using a *NEC1* specific probe. One putative insertion mutant (plant TT249) was identified, with co-ordinates in row 1, column 10 and block 22.

(C) dTph1 insertion in different branches of plant TT249, as detected by PCR and by hybridization, using *NEC1* and transposon specific primers. Wt, wild type (W115); B1-B7, branches 1 to 7 of TT249. Branches B1-B5 still contain the dTph1 insertion.

(D) dTph1 insertion in F1 plants of W115 × TT249 as detected by PCR and hybridization, using *NEC1* and transposon specific primers. Plants 1, 7, 9, 19 and 23 contain one *dTph1* insertion in *NEC1*, plant 3 contains two *dTph1* insertions.

The occurrence of two highly similar genes of *NEC1* was further verified by Southern blot analyses of the F3 population after selfing of F2 *dTph1* plants (a^T_1 $a^T_1A_2$ A_2). As expected, all the plants of the F3 population were homozygous for the insertion in one of the two highly similar genes of *NEC1*. All plants have two strong hybridization bands of 1.1kb and 1.4kb (Figure 5D) and the "early open anther" phenotype was observed in all plants. When F2 *dTph1* plants (a^T_1 $a^T_1A_2$ A_2)

were crossed with W115 ($A_1A_1A_2A_2$), all the F1 plants showed a hybridization pattern as expected for $A_1a^T{}_1A_2A_2$ heterozygous insertion plants, the smallest fragment (1.1kb) being stronger than the upper fragment (1.4kb) (Figure 5F).

A highly homologous gene of *NEC1*, *NEC2*, is present in the *Petunia* genome

Alignment of the genomic sequence with that of the *NEC1* cDNA (Ge *et al.* 2000) revealed that the *NEC1* gene contains six exons and five introns, spanning a region of around 2.2kb (Figure 6A). Sequence analysis revealed that there was one internal *HindIII* site in the first intron of *NEC1* (Figure 6A). The presence of the internal *HindIII* site was used to generate 3' and 5' probes for Southern hybridization. Genomic DNA of wild type *Petunia* W115, amplified by PCR using the *NEC1* gene specific primers Prat129 and Prat122 (Figure 6A) yielded a 1964bp fragment, which itself yielded a 211bp (5') and 1753bp (3') fragment after digestion with *HindIII*. These fragments, as well as the full-length cDNA of *NEC1*, were used as probes to hybridize a Southern blot of *HindIII*-digested *Petunia* genomic DNA. Figure 6B shows that hybridization with both the full-length cDNA and the 3' probe gives the same two fragments, while the small 5' probe shows numerous non-specific bands. The results confirm the presence of a *NEC1*-like gene, further referred to as *NEC2*.

dTph1 insertion is in an exon of *NEC1*

Southern blot hybridization was performed on plants homozygous for the transposon insertion ($a^T{}_1a^T{}_1A_2A_2$, see figure 5B). After digestion with *HindIII* both wild type and homozygous insertion plants show two hybridizing bands (Figure 6C). However, the upper hybridizing fragment in the insertion plant is ± 300bp larger compared to the upper band of wild type, indicating the *dTph1* insertion to be present in the upper fragment.

To determine if the *dTph1* insertion is in *NEC1* or *NEC2*, a PCR reaction was carried out on genomic DNA of the homozygous insertion plant, using *NEC1* gene-specific primers p166 and p122 (Figure 6A). A ± 1000bp and a ± 1300bp fragment were amplified and cloned. Sequence analysis showed that the 1300bp fragment contained the *dTph1* inserted in an exon of *NEC1* (Figure 6A). The sequence data on the 1000bp fragment showed that no transposon insertion was present, the sequence showing high (95%) homology to the sequence of the *NEC1* fragment (data not shown). It was concluded that the 1000bp fragment represents a 3'-terminal fragment of *NEC2*, while the *dTph1* insertion is in *NEC1*.

Discussion

NEC1 is a gene that is highly expressed in nectaries of *Petunia*, while very localized expression is also observed in the anther stomium and in the tip of the anther filament (Ge *et al.* 2000). The results of over-expression of *NEC1* and of localization of the protein with transmission electron microscopy (TEM) in nectaries, suggested its role to be in sugar metabolism and nectar secretion. However, over-expression of *NEC1* did not result in a mutant nectary or anther phenotype. In this chapter, we show that partial down-regulation of *NEC1* results in a phenotype in the anthers, but not in the nectaries. Complete down-regulation was not achieved due to the indicated presence of a *NEC1*-like gene, nominated *NEC2*, which shows very high homology to *NEC1*.

Figure 5. Segregation analysis of progeny from the cross of W115 × TT249.

(A) Expected pedigree of F2 progeny for subsequent molecular genetic analysis if *NEC1* gene is a single copy gene. "A" stands for *NEC1* gene, "a^T" stands for *dTph1* insertion in *NEC1* gene. HP, hypothetical hybridization pattern.

(B) Expected pedigree of progeny for subsequent molecular genetic analysis in two copies or highly homologous genes are present in genomic DNA. "A_1" and "A_2" stand for the *NEC1* and a *NEC1*-like gene, "$a^T{}_1$" stands for *dTph1* insertion in *NEC1* genes. HP, hypothetical hybridization pattern.

(C) DNA gel blot analysis of F2 progeny digested with genomic DNA of *NEC1* insertion mutant.

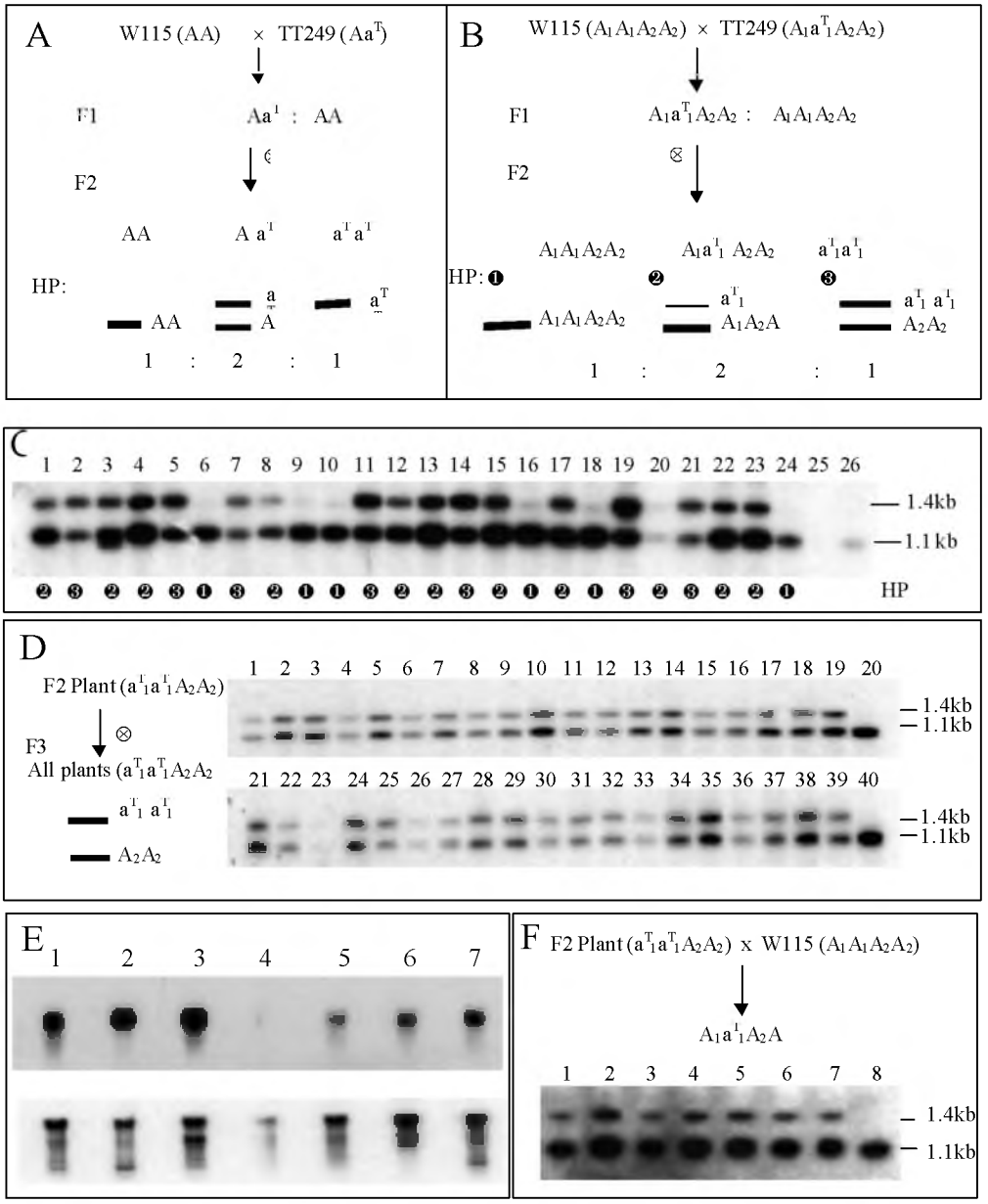
Genomic DNA was digested with *Nco1* and *EcoRV* (Fig. 6A) and the gel blot was hybridized with a *NEC1* specific probe. There are two hybridized bands, in which one band (1.1kb) is about 300bp smaller than the product from the *dTph1* insertion band (1.4 kb). Lanes 1-24 are the progeny plants and lanes 25, 26 are wild type controls. ❶, ❷ and ❷ stand for three kinds of hybridization patterns as shown in **(B)**.

(D) DNA gel blot analysis of F3 progeny from F2 pants ($a^T{}_1$ $a^T{}_1A_2$ A_2). Lanes 1-19 and 21-39 are progeny plants, lanes 20 and 40 are wild type controls.

(E) RNA expression levels of *NEC1* in nectaries of different progeny plants. Filter was hybridized with full-length [^{32}P]-labeled *NEC1* cDNA (top) and a ribosomal DNA probe (bottom).

Lane 1: wild type control, lanes 2 and 3: plants (A_1 A_1A_2 A_2), lanes 4 and 5: plants ($a^T{}_1a^T{}_1A_2$ A_2) and lanes 6 and 7: plants ($A_1a^T{}_1A_2$ A_2).

(F) DNA gel blot analysis of the progeny from W115 (A_1 A_1A_2 A_2) × F2 pants ($a^T{}_1a^T{}_1A_2$ A_2). Lanes 1-7 are the progeny plants and lane 8 is the wild type control.



Transposon mutagenesis

In the present study we used transposon insertion alleles to establish the loss-of-function phenotypes for the *NEC1* gene. Previously, it was suggested that virtually any gene in the *Petunia* genome could be inactivated by a *dTph1* insertion, the insertion alleles for various genes occurring at a frequency of about 1 in 1000 plants (Koes *et al.* 1995). In this study among 2410 plants were screened, one plant TT249 was isolated, which showed a *dTph1* insertion in the protein coding sequence of *NEC1*. This plant was heterozygous for the insertion allele. After selfing, a progeny was obtained, which contained plants that were homozygous for the insertion in *NEC1*, but had no insertion

in *NEC2*. Such plants showed the "early open anther" phenotype, however, no aberrant nectar phenotype was observed. It is assumed, therefore, the gene product of *NEC* is redundant. Even when *NEC1* is knocked out by homozygous *dTph1* insertion, *NEC2* is still functioning. Apparently, *NEC2* gene expression is enough to support normal nectary development and secretion of nectar, but not sufficient to restore a wild type like anther development.

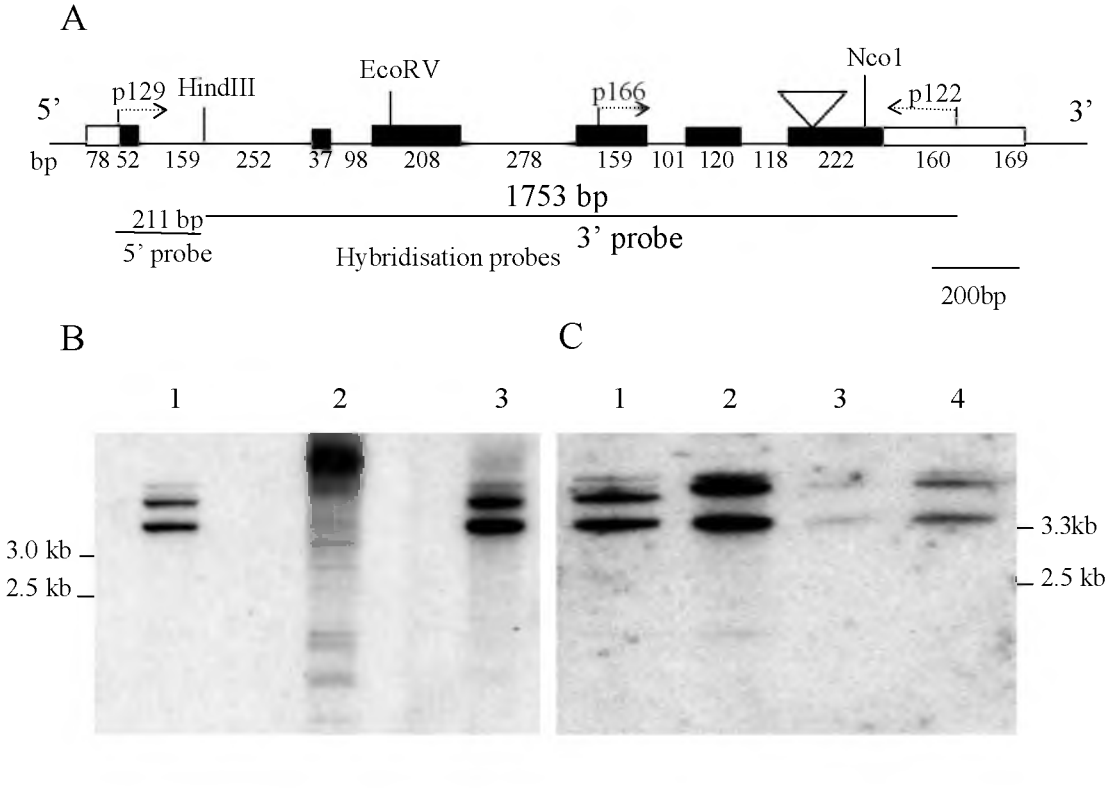


Figure 6. Analysis of the copy number of *NEC1* in *Petunia hybrida*.

(A) Diagrammatic representation of the *NEC1* gene structure. The boxes indicate the six exons (solid boxes for coding regions, open box for untranslated regions), and the lines represent introns. Sizes of the figures are indicative for the length of the fragments in bp. A *dTph1* insertion as detected in TT249 and offspring is indicated by a triangle in the sixth exon of the *NEC1*.

Black bars of 5' probe and 3' probe beneath, indicate the DNA fragments used as hybridization probes in DNA gel blots. *NEC1* gene specific primers Prat 129, Prat 166 and Prat 122 are indicated.

(B) Genomic DNA of W115 was digested with restriction enzyme *HindIII* and fractionated in three lanes of agarose gel. Gel blots of the lanes were hybridized separately with [^{32}P]-labeled *NEC1* cDNA (lane 1), 5' probe (lane 2) and 3' probe (lane 3).

(C) Genomic DNA was digested with restriction enzyme *HindIII*, using the full-length [^{32}P]-labeled *NEC1* cDNA as a probe for hybridization. Lane 1, wild type control; lanes 2-4, independent *dTph1* F2 plants ($a^{T}{}_{1}a^{T}{}_{1}A_{2}$ A_{2}).

The role of *NEC1* in anther dehiscence

During anther dehiscence (anthesis), the anther locules open by rupture of the stomium cells. This process is induced by swelling of the epidermis and the endothecium of the anthers (Keijzer and Willemse 1988). Next, cells of the epidermis and endothecium lose most of their water and shrink, by which the locule walls bend outwards and the anther opens to release the pollen (Keijzer 1987). The molecular regulation of the process of anther dehiscence has not yet been elucidated. Several genes have been suggested to play a role in this process. Stadler *et al.* (1999) postulated that the physiological function of the *AtSUC1* gene is the accumulation of sucrose within a ring of parenchyma cells surrounding the connective tissue, triggering controlled anther dehiscence. Previously, we showed that the expression of *NEC1* is precisely confined to the upper part of the filament and the stomium cells in transgenic plants both of *Petunia hybrida* and *Brassica napus* (Ge *et al.* 2000). In the present study we show that when *NEC1* gene function was partially inactivated, the anther locules opened prematurely. The physiological role of *NEC1* may be in the regulation of the water potential in the stomium or tissues that are supplied with sugars through the anther filament.

Petunia plants that expose the "early open anther" phenotype closely resemble the phenotype that was described earlier for tobacco by Beals and Goldberg (1997), who showed that ablation of the stomium leads to anthers that fail to dehisce. The anther locules bend inwards and no vital pollen was observed. In *NEC1 Petunia* mutants the stomium did not develop normally, having fewer and degenerated cells compared to wild type. Our results confirm that a functional stomium is required for anther dehiscence and *NEC1* thus plays a major role in the regulation of this process.

Detection of phenotypes in nectaries

It was expected that down regulation of *NEC1* through co-suppression or transposon *dTph1* mutagenesis could lead to an altered phenotype in nectaries. The absence of an altered phenotype in nectaries by *dTph1* mutagenesis may first be explained by the presence of two very homologous genes: *NEC1* and a *NEC1*-like gene, *NEC2*. *dTph1* insertion in *NEC1* only resulted in down-regulation of *NEC1*, while *NEC2* was still active. The reason why no phenotype was observed in *NEC1* co- suppression plants may be due to the fact that *NEC1* co-suppression was achieved under the control of the *CaMV 35S* promoter, which does not express in nectaries. The results indicate that the promoter expression pattern of a co-suppression construct affects the phenotype of transgenic plants.

The results in the present study are an important step forward in the elucidation of the molecular regulation of the process of anther dehiscence. *NEC1* appears to play an essential role in the regulation of anther opening and pollen maturation. It was suggested previously that *NEC1* might be involved in nectar secretion, however, its exact role with respect to nectary development and nectar secretion still has to be assessed. The occurrence of two highly homologous genes *NEC1* and *NEC2*, presses the importance of the *NEC* gene products in these processes.

Experimental procedures

Plant materials

Petunia hybrida plants of wild type W115 and the transgenic plants were grown under normal greenhouse conditions.
Four permanent *Petunia* libraries (A1-A4) of *dTph1* insertions were used from wild type W138 available at Plant Research International.

In vitro pollen germination assays

The pollen germination abilities of wild type and co-suppression mutant plants were tested by incubation in a germination medium for five hours, followed by microscopic observation.

Germination medium (500 ml): 50ml 10X salt stock solution (1g/L H_3BO_3, 7g/L $CaNO_3$, 2g/L $MgSO_4$ and 1g/L KNO_3), 10ml 1M MES-buffer pH6.0 (19.52g MES in 100 ml H_2O, pH adjusted to 6.0 with 5M KOH), 10g sucrose and PEG 4000, filter sterilized.

RNA and DNA gel blot analyses

Total RNA was isolated from *Petunia* nectaries or anthers according to Verwoerd *et al.* (1989). Plant DNA was isolated from *Petunia* leaves, according to Koes *et al.* (1986). For RNA gel blot analysis, 10μg of total RNA was denatured by glyoxal (1.5 M) before electrophoresis. Equal loading of RNA in the gel slots was verified by ethidium bromide staining of the gel. For DNA blot analysis, 10 μg DNA was digested with *EcoRV* and *Nco1*, electrophorezed, and blotted onto Hybond N^+ membranes (Amersham).

Membranes were hybridized under standard conditions at 65 °C, with [^{32}-P]-labeled *NEC1* cDNA probes as described by Angenent *et al.* (1992). Normally, the hybridized blot was exposed for one day to X-ray film. The reduction of RNA

transcript by Northern was analyzed in TINA version 2.10h software ©Copyright 1994 raytest Isotopenmeβgeräte GabH.

Gene constructs and plant transformation

*NEC1*p-GUS: the GUS gene was cloned downstream of the *NEC1* promoter (Ge *et al.* 2000).

Co-suppression construct : the full-length cDNA of *NEC1* was cloned downstream of the *CaMV 35S* promoter in sense orientation (Figure 3A). The construct was ligated into the binary vector pBINPLUS (Van Engelen *et al.* 1995) that was transferred to *Agrobacterium tumefaciens*.

Plant transformation was performed as previously described by Ge *et al.* (2000). The transgenic nature of the plants was verified by DNA gel blot analysis and hybridization.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

DNase treatments were carried out on total RNA from nectaries and stamens, using the RNA MessageClean^{TM} Kit (Genhunter Corporation Brookline USA, cat. No. M601). The 1:1000 dilutions of cleaned RNA samples were quantified by OD260. 0.2 µg RNA of each sample was used for reverse transcription (RT), using the oligo-dT primer T12MG from the Genhunter Kit. 1.0 µl of each RT reaction was used as template for PCR amplification with *NEC1* gene specific primers Prat 166 (5'-GGGAGCCCTAGGAATGGTGATGCC-3') and Prat 122 (5'-GTGGGAAGGCTATGCTACAAGC-3') (Figure 2A). The conditions for PCR amplification and hybridization, using full-length [^{32}-P]-labeled *NEC1* cDNA as a probe, were performed as previously described by Ge *et al.* (2000).

Detection of *dTph1* Insertion Alleles

For the detection of insertion alleles in 2410 plants from the four permanent *Petunia* libraries (A1-A4) of *dTph1* insertions available at Plant Research International, the oligodeoxynucleotide transposon element *dTph1* primers TP1 (Prat 54: 5'-CGGAATTCCTGGCTCCGCCCCTG) and TP2 (Prat 55: 5'-CGGAATTCCACCAAGTAGCTCCGCCCCTG) were used, together with the *NEC1* gene specific primers Prat 119 or Prat 129 (Ge *et al.* 2000). PCR was carried out using 30 cycles, each consisting of 30 sec at 94°C, 1 min at 50 °C and 2 min at 72°C (extension 3 sec each cycle). Amplification products were separated by electrophoresis in 1% agarose gels, blotted to hybond-N membranes and hybridized with [^{32}-P]-labeled *NEC1* cDNA probes as described by Angenent *et al.* (1992).

Microscopy

For light microscopic analysis, plant material was fixed, sectioned and stained according to Angenent *et al*. (1993).

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CHAPTER 5

IMPAIRED NECTARY DEVELOPMENT AND STRONGLY REDUCED NECTAR SECRETION IN *PETUNIA HYBRIDA* BY ANTISENSE INHIBITION OF *NEC1*

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Summary

A novel gene *NEC1* was isolated from *Petunia hybrida*, which was highly expressed in nectaries, the anther stomium and the top of the anther filament. To elucidate the function of *NEC1*, the antisense inhibition approach was carried out to down-regulate *NEC1* activity in transgenic *Petunia* plants. Down-regulation of *NEC1* expression resulted in a distinguishable nectary phenotype characterized as impaired nectary development and markedly reduced nectar secretion activity. Furthermore, the antisense plants displayed the similar "early open anther" phenotype, as described for co-suppression and transposon insertion phenotypes. In transgenic *Petunia* plants that expressed the *Barnase* gene under the control of the *NEC1* promoter, cell ablation was observed at an early stage of nectary development. For the first time, nectariless flowers were obtained by targeted, specific cell ablation by *Barnase*. Previous results, together with those presented in this chapter, indicate that *NEC1* plays an important role in two floral developmental processes, namely nectar secretion and anthesis. NEC1, presumably as an osmoregulator, is involved in nectary development and nectar secretion.

Keywords: *NEC1* gene, antisense inhibition, *Barnase* gene, nectary, nectar secretion, *Petunia hybrida*.

Introduction

Nectaries are nectar-secreting organs of plants that are located inside the flowers (floral nectaries) or on vegetative structures outside the flowers (extrafloral nectaries) (Rogers, 1985; Fahn, 1988). The main function of floral nectar is to attract and reward pollinating insects (Baker and Baker, 1975). The function of extrafloral nectaries is less clear, but they probably have a role in plant defense. For example, ants that visit extrafloral nectaries prevent that a plant is attacked by predators (Bentley, 1977; Rogers, 1985).

Floral nectar is a sugarous fluid that mainly consists of sucrose, fructose and glucose. The ratio between different sugars within a species is relatively constant (Baker and Baker, 1982), however, the sugar concentration and nectar production can vary with the age of flowers and external growth conditions of the plants (Shuel, 1961; Free, 1970). Other substances that have been detected in nectars from different plant species include small amounts of other sugars (Baker and Baker, 1982), amino acids (Baker and Baker 1973), proteins (Peumans et al., 1997; Carter et al., 1999) and secondary metabolites like terpenes, alkaloids, flavonoids, glycosides, vitamins, phenolics and volatiles (Baker and Baker 1975; Roschina and Roschina, 1993).

Anatomically, floral nectaries are attached to the base of the filament, the petal, the ovary or other floral organs (Brackenburry, 1995; Galetto, 1995; Link, 1992). They form outgrowths on the surface of these organs like the nectaries of Petunia and Brassicaceae, but they may also be sunken inside the organ, like nectaries of cotton. The ultrastructure of nectaries has been studied extensively, revealing the presence of numerous plasmodesmata between nectary parenchyma cells (Fahn and Rachmilevitz, 1970; Fahn and Benouaiche, 1979). In addition, the endoplasmatic reticulum is often highly developed and, at the stage of secretion, the ER is associated with vesicles that seem to be in contact with the plasmalemma (Fahn, 1979; Eleftherious and Hall, 1983). In some plants, just before nectar secretion, the amount of starch grains in nectaries is very large and declines at the stage of secretion (Zer and Fahn, 1992).

A hypothesis for the mechanism of sugar accumulation and nectar secretion has been postulated by Fahn et al (1979). According to this model, sugar transport to the nectaries is achieved by active transport mechanisms (e.g. sucrose transporters) and a combination of chemical and osmotic gradients. The imported sucrose is partly converted to fructose and glucose, establishing a chemical gradient. Next, part of the glucose is converted to starch establishing an osmotic gradient. Prior to anthesis, starch is hydrolyzed and the accumulated sugars are transported through the plasmodesmata of the nectary parenchyma tissue to nectar secreting cells.(Fahn et al., 1979). The secretion of nectar follows fusion of vesicles that originate from the ER or Golgi with the plasmalemma.

Nectar composition and nectary structure have been studied extensively, however, the molecular control of nectary development and nectar secretion has received little attention. Incidentally, expression studies revealed that the gene described also showed expression in nectaries, such as *CRT*, *CRABS CLAW* and *AGL1* genes in *Arabidopsis thaliana* (Bowman and Smyth, 1999; Flanagan *et al.*, 1996; Nelson *et al.*, 1997), a *myb* gene in *Antirrhinum* (Jackson *et al.*, 1991), the MADS box gene pMAD3 in petunia (Kater et al., 1998) and *NTR1* gene in *Brassica campestris* L. *ssp. pekinensis* (Song *et al.*, 2000). However, these studies did not reveal the function of such genes in nectary development or nectar secretion.

Recently, a study directed towards the elucidation of the molecular regulation of nectary development and nectar secretion in *Petunia hybrida* has been undertaken (Ge *et al.* 2000, 2001a). We cloned a novel gene, *NEC1*, which is highly expressed in nectary tissue of *Petunia hybrida* and in lateral nectaries of *Brassica napus* (Ge *et al*, 2000). In addition, *NEC1* is expressed in the upper part of the anther filament and in the anther stomium. Down regulation of *NEC1*, either by co-suppression or transposon mutagenesis, affected anther development, but did not result in a mutant nectary phenotype (Ge *et al.*, 2001a). The analysis of the so-called "early open anther" phenotype showed that *NEC1* appears to play a role in the development and opening of the stomium (Ge *et al.*, 2001a). The absence of an altered phenotype in nectaries by

transposon mutagenesis was probably due to the presence of a highly homologous gene, *NEC2*. The *dTph1* insertion only resulted in down-regulation of *NEC1*, while *NEC2* was still active. The absence of a nectary phenotype in *NEC1* co-suppression plants was interpreted as being the result of using a promoter (*CaMV 35S*) that is not active in nectaries.

An alternative way to obtain down-regulation of a gene is antisense inhibition. Previously, antisense genes have been successfully used to silence genes that are involved in plant processes such as flower pigmentation, fruit ripening and photosynthesis (Fray and Grierson, 1993; de Lange *et al.*, 1995). The present article reports on the phenotypes of nectaries and anthers as affected by antisense inhibition of *NEC1* gene expression under the control of a nectary-specific promoter.

In addition, the effects of tissue-specific cell ablation through *NEC1* promoter targeted expression of *Barnase* are presented. *Barnase* is a very active extracellular ribonuclease that was cloned from *Bacillus amyloliquefaciens* (Paddon and Hartley, 1986). The *Barnase* gene can be applied to ablate specific tissues as was e.g. demonstrated in the anthers of tobacco (Mariani *et al.*, 1990.

The results presented in this chapter reveal the function of a novel gene *NEC1* in nectar production. On basis of a model it is explained which step during the process of nectar production is regulated by the expression of *NEC1*.

Results

Down regulation of *NEC1* expression inhibits starch hydrolysis in nectaries and nectar secretion

To inhibit *NEC1* gene expression in *Petunia* plants, we followed the antisense gene approach. A binary vector containing the full-length cDNA of *NEC1* in antisense orientation, downstream of the *NEC1* promoter (Figure 1A) was introduced into *Petunia hybrida* line W115. Out of 30 independent transformants, seven transgenic *Petunia* plants that showed an aberrant nectary phenotype were obtained. DNA gel blot analysis revealed that these plants were transgenic (data not shown). The nectaries of these transgenic plants showed tissue degeneration and anthocyanin pigmentation in the outer nectary parenchyma cells, the extension of degeneration varying from groups of cells in weak phenotypes to large encavements in severe transformants (Figure 1C, D). However, the phenotype was only observed in flowers after anthesis, during the stage that nectar secretion is most abundant. In addition, it was observed that in plants with a mutant nectary phenotype, on average 3 anthers per flower already opened, while the flower buds were still closed (Figure 1E-H). This is in contrast to wild type

Petunia plants, where the anthers open when the pollen is mature and the flowers are open. This antisense phenotype is very similar to the "early open anther" phenotype as previously described for co-suppression and transposon insertion mutagenesis of *NEC1* (Ge *et al.*, 2001a). In addition, in the weak phenotype of *NEC1* antisense plants, anthocyanin pigmentation was observed in the anther stomium and the upper part of the anther filament (Figure 1K-L). The anthocyanin pigmentation pattern in the anthers exactly coincides with the location of GUS activity, driven by the *NEC1* promoter (Figure 1I, J).

To assess down-regulation of *NEC1* expression, Northern blot analysis was performed on nectaries of a selection of primary transformed plants (Figure 2A). In plants showing a severe nectary phenotype (lanes 2, 5, and 7), *NEC1* RNA expression was reduced almost completely. In plants with a weak phenotype (lanes 4, 8, 9 and 11), *NEC1* RNA expression was reduced between 60% and 90%. The level of down-regulation of *NEC1*, thus corresponds to the severity of the nectary phenotype.

A detailed phenotypic analysis of antisense transgenic line T127001 was performed using scanning electron microscopy (SEM) and histological examination. It was shown that in wild type plants, the surface of the nectaries of flowers after anthesis was regular and smooth, while nectar was secreted from the outer nectary cells (Figure 2B, C). Comparatively, the nectary surface from mutant plants was rough and irregular, while limited nectar was secreted (Figure 2D, E).

Histological sections through nectaries of wild type flowers and those of *NEC1* antisense plants were made to examine the nectary phenotype at the cellular level (Figure 3). Sections were made through nectaries of flowers just after anthesis when the nectary phenotype in antisense plants was most severe. In wild type plants this is the stage at which starch that has accumulated during nectary development is rapidly hydrolyzed and nectar secretion is very abundant. Sections through the middle part of a wild type nectary show regular nectary parenchyma cells with dense cytoplasm (Figure 3A, G). Interestingly, at the location where cell degeneration takes place in nectaries of the antisense plants, nectary cells contain almost no cytoplasm and the nuclei appear as shrunken dark spots (Figure 3B, H). The onset of cell degeneration can be observed at the inner periphery of tissue regions, where many nectary parenchyma cells are undergoing plasmolysis (Figure 3F). In wild type nectaries, no plasmolysis is observed and intercellular spaces are visible between the nectary parenchyma cells (Figure 3E). A specific starch staining was performed to examine if starch accumulation or hydrolysis in the nectary mutant is aberrant. In wild type nectaries, starch has been hydrolyzed almost completely, which is the normal process for *Petunia* nectaries in flowers after anthesis (Figure 3C, I). In contrast, nectaries of antisense plants at this stage still contained abundant starch (Figure 3D) with numerous large amyloplasts (Figure 3J).

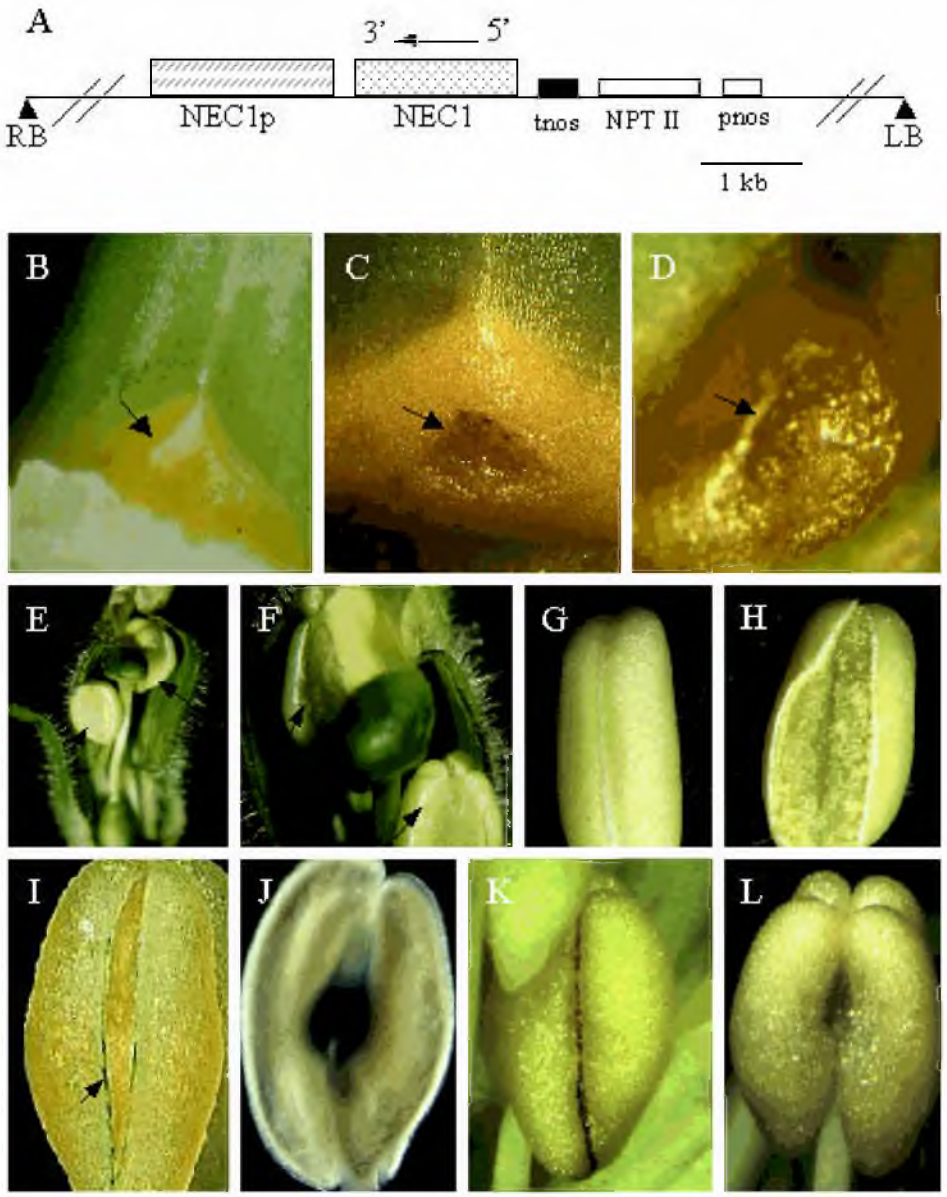


Figure 1 Analysis of nectary and anther phenotypes as affected by *NEC1* antisense inhibition in *Petunia hybrida.*

(A) Antisense construct of *NEC1*: the full-length *NEC1* cDNA was inserted in the antisense orientation downstream of the *NEC1* promoter (*NEC1*p) and upstream of the nopaline synthase terminator (Tnos). NPTII, neomycin phosphotransferase II gene; pnos, nopaline synthase promoter; tnos, nopaline synthase terminator; RB, right border; LB, left border.

(B) Wild type nectary (arrow) on the basis of the ovary.

(C, D) Impaired nectary development in different *NEC1* antisense independent transformants, showing tissue degeneration and anthocyanin pigmentation in the outer nectary parenchyma cells.

(E, F) Arrows indicate opened stomium in anthers of "early open anther" phenotype in small flower buds (2cm) in *NEC1* antisense line T127001.

(G, H) Anthers in closed flower buds (5 cm) at stage 2 (Ge *et al.*, 2000) from wild type **(G)** and from *NEC1* antisense line T127001 **(H)**.

(I, J) GUS activity driven by the *NEC1* promoter, showing GUS staining (arrow) in the anther stomium **(I)** and the top of the filament **(J)**.

(K, L) Anthers of *NEC1* antisense inhibition line T127015, showing anthocyanin pigmentation in the anther stomium **(K)** and the upper part of the filament **(L)**.

Nectariless flowers by targeted expression of *Barnase*

The activity of the *NEC1* promoter was monitored by expressing the bacterial *Barnase*. The T-DNA region of the *NEC1-Barnase-Barstar* construct used for plant transformation is shown in Figure 4A. The effect of expression of this cytotoxic gene was analyzed in 52 independently generated primary *Petunia* transformants. Sixteen plants showed developmental defects in nectaries and anthers, the severity of the phenotype varying considerably among transformants. Southern blot hybridization confirmed that two to three copies of the transgene were present in the transformants showing a phenotype (data not shown). Eleven of 16 plants had a weak phenotype, displaying only some black spots on the nectary surface (Figure 4D). These plants were not analyzed further. Two major groups of transformants were identified from the five plants showing a severe phenotype. In the first group, nectaries were completely eliminated (Figure 4C). In the second group, necrosis of nectary tissue and progressive browning was observed at a later stage of nectary development (Figure 4D-G). The nectaries of these plants developed normally until the stage 3 (Ge *at al.*, 2000), just before anthesis, when necrotic cells became visible as dark spots on the surface of the nectary (Figure 4D). During further floral development, progressive browning and necrosis of the nectary tissue took place, affecting also the ring of nectariferous tissue around the carpel base (Figure 4F, G). The effect of *Barnase* in the anthers was exhibited as browning in the region of the stomium (Figure 4H) and shrinking of the upper part of the filament (Figure 4J). The location of *Barnase* activity in the stamen has exactly coincided with GUS activity, as driven by the *NEC1* promoter (Figure 1I, J).

To analyze the effects of *Barnase* activity on the morphology of nectaries, we performed SEM analysis on the second group with a severe phenotype, characterized by necrosis and progressive browning of nectary tissue (Figure 5).

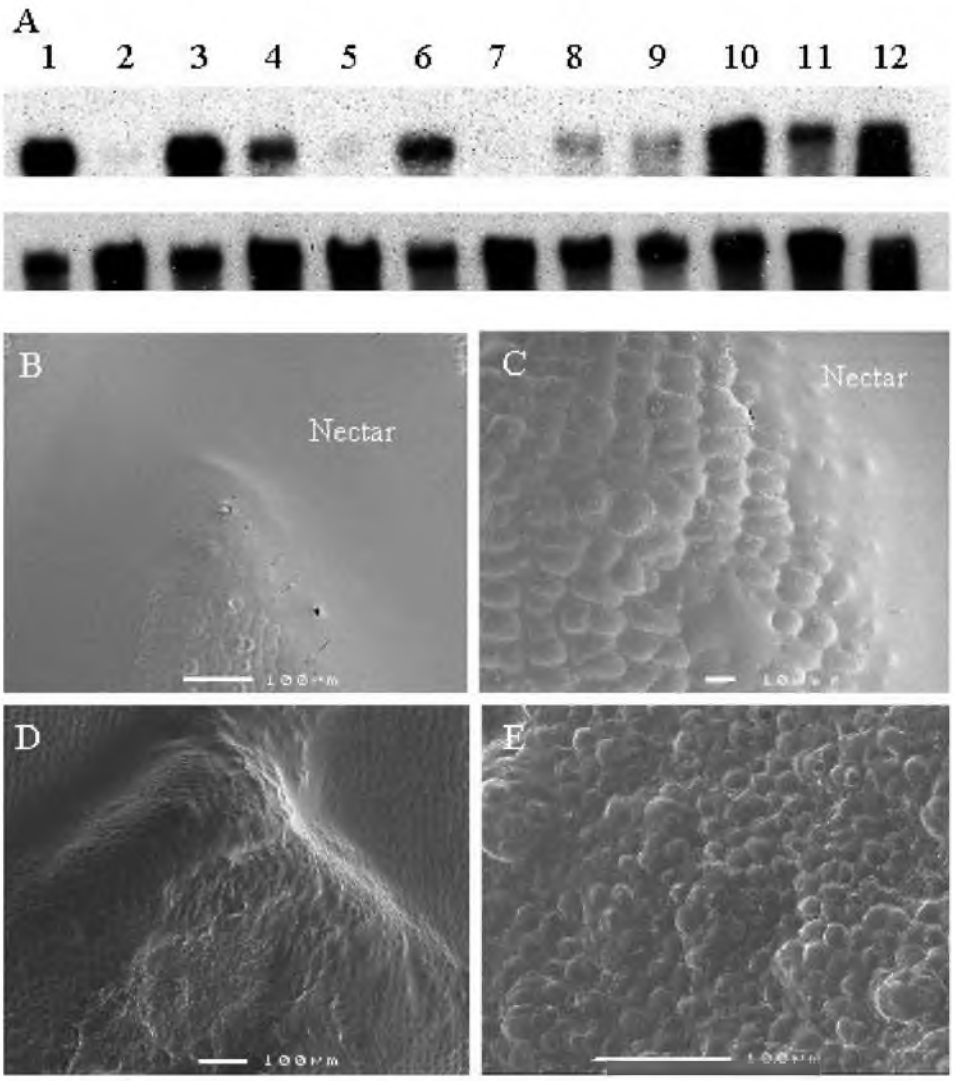


Figure 2. Analysis of *NEC1* expression and nectary phenotype of *NEC1* antisense inhibition transformants.

(A) *NEC1* RNA expression in nectaries of independent primary transformants. RNA gel blot was hybridized with full-length [^{32}P]-labeled *NEC1* cDNA (top), and ribosomal DNA probe (28S rRNA, bottom). Lane 1: wild type (W115). Lanes 2-12, a selection of primary transformed plants, T127001, T127002, T127009, T127010, T127011, T127012, T127015, T127016, T127017, T127018 and T127020.

(B, C) Scanning electron microscopic (SEM) analysis of nectaries from wild type (W115) flowers after anthesis. The surface of the nectaries is regular and smooth with abundant nectar. Bar = 100 μm **(B)**, bar = 10 μm **(C)**.

(D, E) SEM analysis of a *NEC1* antisense inhibition plant T127001 after anthesis. The surface of the nectaries is rough and irregular without nectar. Bar = 100 μm.

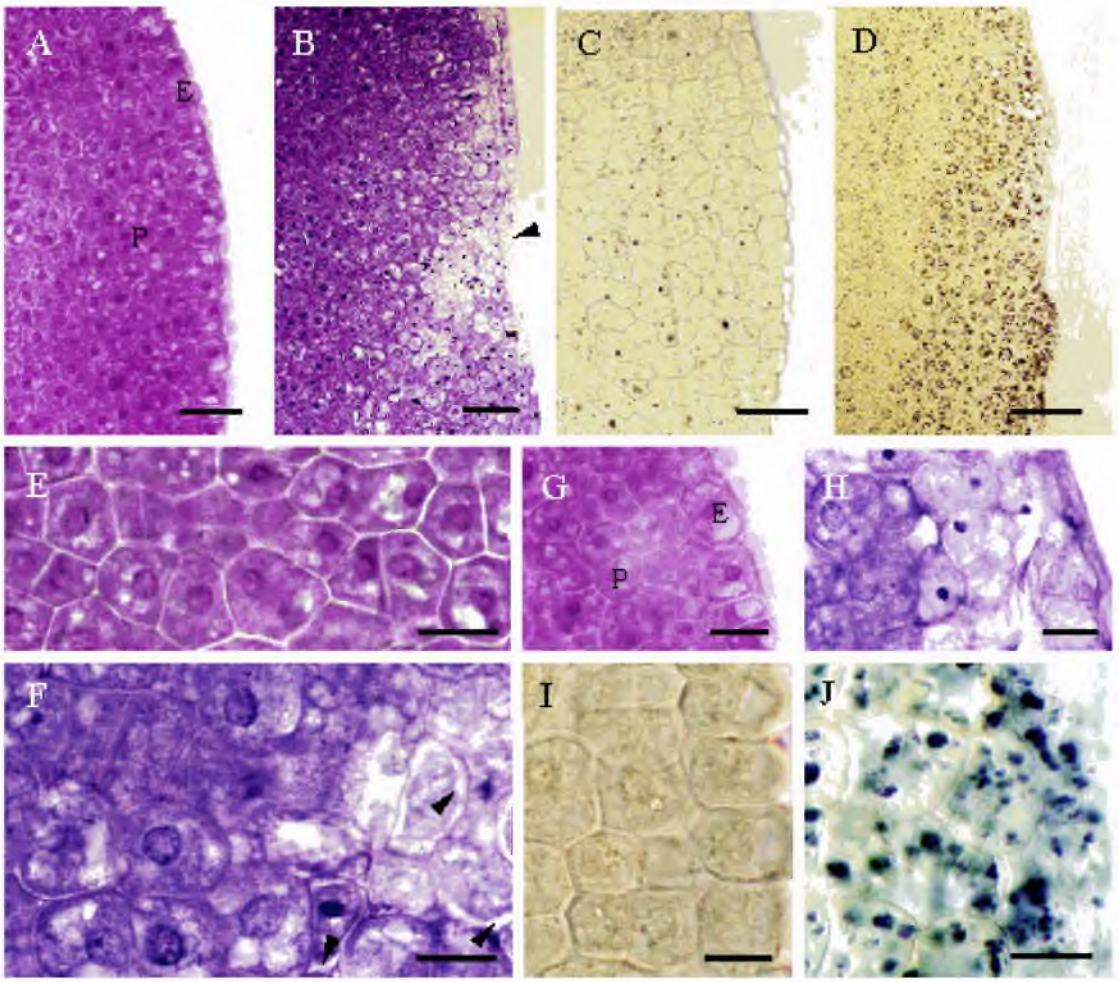


Figure 3. Histological analyses of nectary cells of *Petunia hybrida* in wild type and antisense transgenic plant T127001. Longitudinal sections were made from nectaries of flowers that are at the stage after anthesis, when in wild type flowers abundant nectar secretion takes place (stage 4).

(A, B) Sections through the outer part of the nectary of wild type **(A)** and the *NEC1* antisense mutant **(B)**, showing cell degeneration in epidermis (E) and in the outer nectary parenchyma (P). Bar = 30 µm.

(C, D) Sections through the outer part of the nectary of wild type**(C)** and the *NEC1* antisense mutant **(D)** were treated with I_2-KI, staining starch dark brown. In wild type nectaries starch has been hydrolyzed almost completely, while in the antisense mutant numerous starch grains are still present in the outer nectary parenchyma cells. Bar = 30 µm.

(E, F) Magnification of the middle part of the sections shown in **(A)** and **(B)**, showing regular nectary parenchyma cells in wild type nectaries **(E)**, with intercellular spaces clearly visible between the cells. In nectaries of the antisense mutant **(F)**, cells adjacent to the site were cell degeneration is taking

taking place are undergoing plasmolysis (arrows) and no intercellular spaces are visible. Bar = 10 μm.

(G, H) Magnification of the sections through the outer nectary parenchyma, adjacent to the site where cell degeneration is taking place. In wild type nectaries cells contain dense cytoplasm and normal stained nuclei **(G)**. In the antisense mutant cells are undergoing plasmolysis and the nuclei are darkly stained and shrunken **(H)**. Bar = 10 μm.

(I, J) Similar sections as shown in G and H, were stained for starch. In wild type nectaries **(I)** no starch is detected in nectary parenchyma cells or the epidermal cells. In the *NEC1* antisense mutant starch has not been hydrolyzed and many starch grains are visible in nectary parenchyma cells and the epidermis **(J)**. Bar = 10 μm.

Figure 4. The effect of *NEC1* promoter driven *Barnase* expression on nectary and anther development.

(A)*NEC1* promoter-*Barnase* gene construct. The *NEC1* promoter was fused to the bacterial *Barnase* and *Barstar* gene. *NEC1*p, *NEC1* promoter; *35S* polyA, cauliflower mosaic virus terminator sequence; NPTII, neomycin phosphotransferase II gene; pnos, nopaline synthase promoter; tnos, nopaline synthase terminator; RB, right border; LB, left border.

(B) Pistil of a wild-type *Petunia* flower at the stage just before anthesis, with nectaries (arrow) and a ring of nectariferous tissue around the base of the pistil.

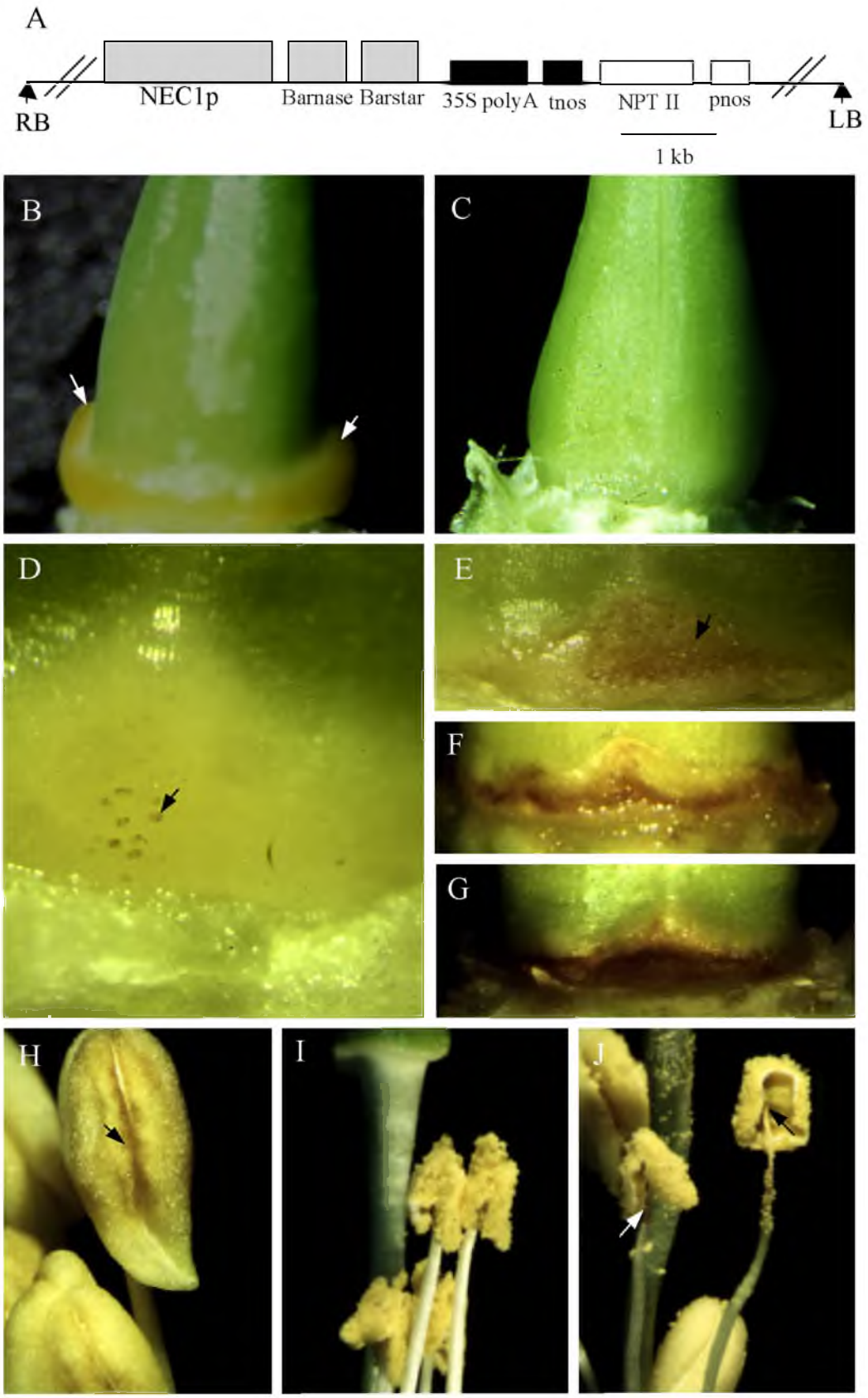
(C) Pistil of a nectariless *Petunia* flower at the stage just before anthesis in transgenic plant T107009. Nectaries as well as the ring of nectariferous tissue are ablated.

(D) Detail of the surface of a nectary from transgenic plant T107002. Flower stage is before anthesis. Several cells at the basis of the nectary show necrosis.

(E-G) Browning and cell ablation in nectaries of transgenic plants that express *Barnase*, progressing from a small region (arrow) in flowers at the onset of anthesis **(E)** to almost the whole nectary at a late stage of anthesis **(G)**.

(H) Anther from a transgenic plant expressing *Barnase*, showing browning alongside the anther stomium. Anthers are from a flower at the stage just before anthesis.

(I, J) The effect of *NEC1* controlled *Barnase* expression on the filament tip. The anther filaments of wild type flowers at the stage after anthesis are straight and regularly shaped **(I)**. The anther filaments affected by *Barnase* **(J)** are curved and show thinning of the filament tip (arrow).


A
RB
NEC1p
Barnase Barstar
35S polyA tnos
NPT II
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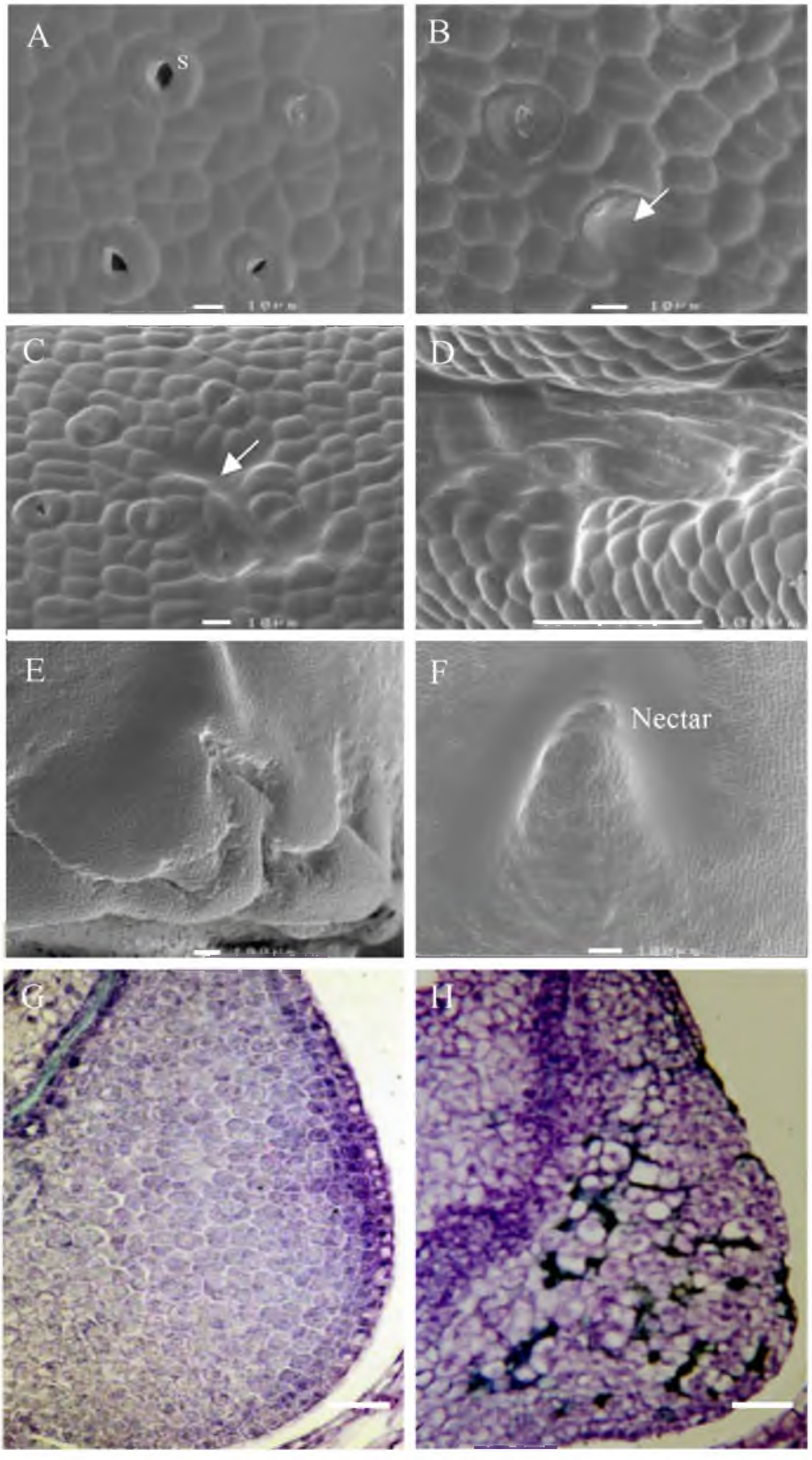
A
S
B
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Nectar
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Figure 5. Scanning electron microscopic (SEM) and histological analysis of nectaries from mutants that express *Barnase* under the control of the *NEC1* promoter.

(A) SEM of wild type nectaries at stage 3 before anthesis. Epidermal cells interspaced with stomatal cells (S). Bar = 10 μm.

(B-E) SEM of nectaries from *NEC1p-Barnase* mutant T107002. During flower development cell ablation progressively occurred from one cell at stage 3, before anthesis **(B)**, to groups of cells during anthesis **(C)** and to larger regions of nectary parenchyma tissue after anthesis **(D, E)**. Bar = 10 μm **(B, C)**, Bar = 100 μm **(D, E)**.

(F) Wild type nectary with nectar secretion, at stage 4, after anthesis. Bar = 100 μm.

(G, H) Longitudinal sections through nectaries from flowers at stage 4, after anthesis. Wild type nectaries are characterized by a regular epidermis and nectary parenchyma cells with dense cytoplasm **(G)**. In nectaries where *Barnase* is expressed cell ablation is visible throughout all nectary parenchyma tissue **(H)**. Bars = 50 μm.

The surface of mature wild type *Petunia* nectaries of flowers before anthesis consists of nectary epidermal cells, interspaced with numerous opened stomata (Figure 5A). In same stage flowers of *Barnase* lines, cell ablation was observed in the outer nectary cells and often appeared to start from a stomatal cell (Figure 5B). In flowers at the stage of anthesis, cell ablation progressed to groups of cells in the outer nectary parenchyma (Figure 5C). In flowers after anthesis large cavities were formed (Figure 5D, E). During and after anthesis, abundant nectar secretion was observed in wild type *Petunia* nectaries (Figure 5F), but no nectar was secreted from nectaries of the severe *Barnase* plants. Histological examination of longitudinal sections through nectaries of plants expressing the *Barnase* phenotype further revealed that cell death also occurred at the inner nectary parenchyma cells (Figure 5H). *Barnase* lines with a severe nectary phenotype showed normal vegetative development, but pollen fertility was low. Only 1-5% of the pollen germinated in vitro (data not shown).

Discussion

Antisense expression of *NEC1* under the control of the *NEC1* promoter was applied successfully to inhibit *NEC1* RNA expression. This has resulted in impaired nectary development and strong to complete reduction of nectar secretion.

Nectar production is defined as the combination of sugar accumulation and nectar secretion. In our previous studies, we have shown that *NEC1* is highly expressed in nectaries of *Petunia hybrida* flowers and that the expression was the highest at the stage after anthesis when active nectar secretion took place (Ge *et al.*, 2000). We

observed that *NEC1* was only expressed in the nectar secreting lateral nectaries of *Brassica napus*, whereas no expression was observed in non-secreting median nectaries (Ge *et al.*, 2000). Therefore, we concluded that *NEC1* plays a role in nectar production. However, no nectary phenotype could be detected by sense co-suppression or transposon insertion in *NEC1* (Ge *et al.*, 2001a). The co-suppression construct used in these investigations comprised the *CaMV 35S* promoter, which was found to be not active in nectaries of *Petunia* (data not shown). Although co-suppression is considered to be independent of the specificity of the promoter (van Blokland *et al.*, 1994; van Der Krol *et al.*, 1990), we concluded that a specific promoter might be needed to down-regulate *NEC1* in nectaries. Several studies have shown that the antisense RNA is only effective if strong promoters are used, which are active in the same cell types as those of the resident genes (Cannon *et al.*, 1990; de Lange *et al.*, 1995; van der Meer *et al.*, 1992).

In the present study we showed that when *NEC1* is expressed in antisense orientation under the control of the strong, nectary-specific *NEC1* promoter, almost complete down-regulation of *NEC1* is achieved. Moreover, *NEC1* down-regulation resulted in a distinguishable nectary phenotype characterized by impaired nectary development and loss of nectar secretion capacity.

These results support our conclusions from previous studies that *NEC1* is a gene that is indispensable for nectar secretion. Detailed microscopic analysis of nectaries from antisense and wild type plants in this study further elucidated the function of *NEC1*. It was shown that tissue degeneration in the antisense plants started at the periphery of nectaries. Plasmolysis in cells surrounding degenerating nectary cells indicates that this process apparently preceded cell degeneration. Generally, plasmolysis in cells will occur when the water potential within the plasmalemma is lower than in the surrounding intercellular fluid. Interestingly, the cells of the outer nectary tissue of the antisense plants were filled with starch, while in wild type nectaries at the same stage starch had been hydrolyzed almost completely. The results indicate that down regulation of *NEC1* inhibits starch hydrolysis in nectaries. Thus, one of the mechanisms of nectar secretion could be that the starch hydrolysis in the outer nectary tissue increases the water potential of these cells, thereby establishing an osmotic gradient from the inner to the outer nectary cells. This osmotic gradient may direct the flow of nectar to the outer nectary cells for secretion, while the released monosaccharides themselves contribute to nectar production. In antisense mutants, starch hydrolysis does not take place and, consequently, an osmotic gradient cannot be established. Thus, nectar secretion is inhibited, while the low water potential of the starch-filled cells induces plasmolysis and finally cell death. The present results unequivocally explain our previous submicroscopic observations, namely that NEC1 protein is abundantly present in the starch grains of nectaries (Ge *et al.*, 2000).

Furthermore, the results are in accordance with our observations that *NEC1* promoter-driven GUS expression was the highest in cells that had just undergone starch hydrolysis (Ge *et al.*, 2000). A model for nectar secretion and the role of *NEC1* is illustrated in Figure 6.

Previous results showed that partial down-regulation of *NEC1* expression resulted in the so-called "early open anther" phenotype (Ge *et al.*, 2001a). An identical phenotype was observed in this study, using antisense inhibition of *NEC1*. In these plants the anthers open prematurely at a stage that flower buds are very small and still closed. The anthers produce no vital pollen and the plants are male sterile. In wild type plants the mechanical process of anthesis is controlled by differential regulation of the osmotic pressure in cells surrounding the anther stomium (Keijzer, 1987). Genes that regulate this process have not yet been described. *NEC1* appears to be at least one of the genes that are involved in this process, and its possible role may be to regulate starch hydrolysis in cells of the anther stomium or surrounding tissues. Further investigations are required to elucidate the exact function of *NEC1* in the process of anther dehiscence.

Petunia plants that express *Barnase* under the control of the *NEC1* promoter showed two phenotypes. In one of the phenotypes, cell death first occurred in solitary cells, and then gradually proceeded to groups of cells and to all the nectary tissue. The gradual progression of cell ablation demonstrates that *NEC1* promoter activity occurs at a late stage of nectary development. However, the occurrence of a completely nectariless phenotype demonstrates that *NEC1* expression may also occur during the initial stage of nectary development.

In conclusion, this study has further elucidated the function of *NEC1* in the important floral developmental processes of nectary development, nectar secretion and anthesis. Furthermore, for the first time, nectariless phenotypes were obtained by targeted expression of *Barnase* in nectaries.

Experimental procedures

Plant materials

Petunia hybrida plants of wild type W115 and the transgenic plants were grown under normal greenhouse conditions.

To study the nectary phenotypes of transgenic plants, four flower stages were distinguished, as described in Ge *et al.* (2000). Briefly, the stages are:

1. Flowers closed, length 3-4cm, nectaries white, no nectar secretion, before anthesis.
2. Flowers closed, length 5-6cm, nectaries light yellow, nectar secretion, before anthesis.
3. Flowers open, length 6- cm, nectaries yellow, nectar secretion, before anthesis.
4. Flowers open, length 6- cm, nectaries orange, nectar secretion, after anthesis.

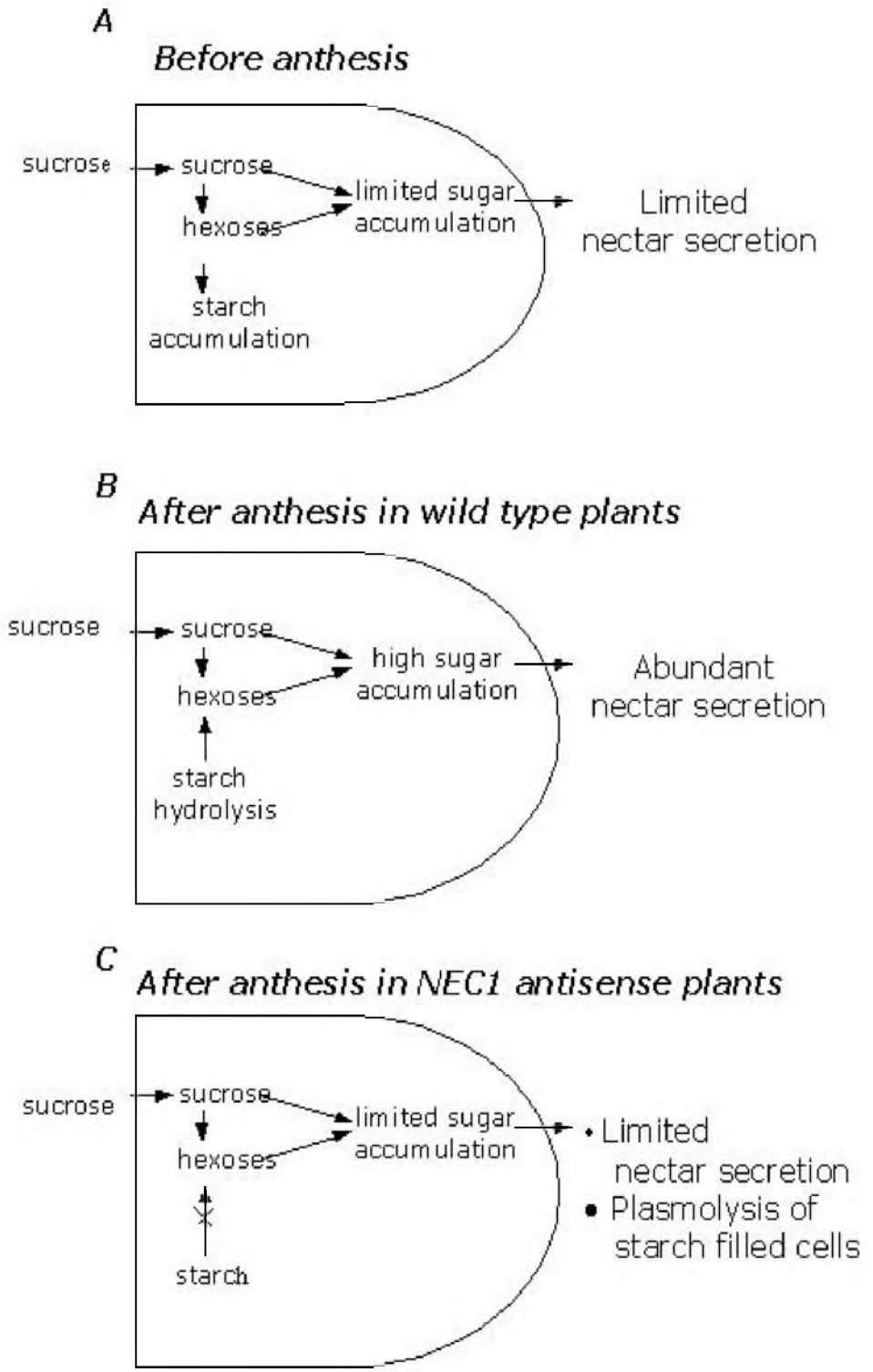

Figure 6. Schematic presentation of a model for the role of *NEC1* in nectar secretion in *Petunia hybrida*.

(A) Phloem-derived sucrose is imported into the nectary parenchyma tissue, apparently by active transport mechanisms. Before anthesis, part of the imported sucrose is converted to fructose and glucose, establishing a chemical gradient for sucrose, facilitating passive import of sucrose. Part the released glucose is converted to starch. A limited amount of nectar is produced, consisting of the remaining sucrose, fructose and glucose.

(B) In wild type plants just before anthesis, *NEC1* affects rapid starch hydrolysis in nectaries, resulting in high sugar accumulation in the outer nectary parenchyma cells. The resulting high osmotic potential of these cells will attract nectar from the inner nectary cells, thus stimulating the flow and secretion of nectar secretion.

(C) As a result of *NEC1* down-regulation, starch is not hydrolyzed, resulting in a low osmotic potential of the outer nectary cells. No osmotic gradient from the inner to the outer nectary cells is established, thus, nectar flow is strongly inhibited. Furthermore, the cells that have accumulated starch plasmolyse and degenerate, leading to further reduction of nectar secretion.

RNA and DNA gel blot analyses

Total RNA was isolated from *Petunia* nectaries or anthers according to Verwoerd *et al.* (1989). Plant DNA was isolated from *Petunia* leaves, according to Koes *et al.* (1986). For RNA gel blot analysis, 10μg of total RNA was denatured by glyoxal (1.5 M) before electrophoresis. Equal loading of RNA in the gel slots was verified by ethidium bromide staining of the gel. For DNA blot analysis, 10 μg DNA was digested with *EcoRV* and *Nco1*, electrophorezed, and blotted onto Hybond N^+ membranes (Amersham).

Membranes were hybridized under standard conditions at 65 °C, with [^{32}-P]-labeled *NEC1* cDNA probes as described by Angenent *et al.* (1992). Normally, the hybridized blot was exposed for one day to X-ray film. The reduction of RNA transcript by Northern was analyzed in TINA version 2.10h software ©Copyright 1994 raytest Isotopenmeβgeräte GabH.

NEC1 gene constructs and plant transformation

NEC1 antisense construct: briefly, the full-length cDNA of *NEC1* (Ge *at al.*, 2000) was inserted in the antisense orientation downstream of the *NEC1* promoter and upstream of the nopaline synthase terminator (Tnos) (Figure 1A).

NEC1-Barnase construct: the *Barnase-Barstar* bacterial operon construct (Hartley, 1988) was cloned downstream of the *NEC1* promoter (Figure 4A).

The *NEC1* antisense and the *NEC1-Barnase* fragments were ligated into a pBIN-derived vector pBINPLUS (Van Engelen *et al.*, 1995). The recombinant vectors were transferred via *Agrobacterium tumefaciens* (LBA4404) to *Petunia* variety W115, using the standard leaf disk transformation method (Horsch *et al.*, 1985). After shoot and root induction on kanamycin (250μg/ml) selection media, plants were transferred to soil in the greenhouse. The transgenic nature of the plants was verified by DNA gel blot analysis.

Microscopy

For histological analysis, plant material was fixed in 5% gluteraldehyde in 0.1 M sodium phosphate buffer for 1-2 hr. and rinsed three times for 10 min in 0.1 M sodium phosphate buffer and dehydrated using a series of increasing alcohol solutions. After fixation, the material was embedded in hydroxyethyl methacrylate Technovit 7100, according to the protocol (Kulzer Histo-tec, Wehrheim, Germany). After embedding, the material was placed in a mould, as described by Colombo *et al.* (1997b). The Technovit blocks were sectioned by microtome. Sections of 7 μm were stained with a solution of 1% toluidine blue in distilled water.

For cryoscanning electron microscopy, samples were mounted on a stub, frozen in liquid nitrogen, coated, and observed as described by Angenent *et al.* (1995b).

Starch staining

Sections were stained for the presence of starch, using I_2-KI solution, containing 0.3 g I_2, 1.0 g KI, in 100ml water.

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CHAPTER 6

GENERAL DISCUSSION:

PROSPECTS FOR APPLICATION OF GENES REGULATING NECTARY DEVELOPMENT AND NECTAR PRODUCTION

Introduction

The aim of this thesis is to elucidate nectary-specific gene expression in relation to nectary function by cloning and characterizing nectary-specific genes. Our ultimate aim is to modify nectar composition, using nectary-specific genes or promoters in order to make flowers more attractive for pollinators or to make the plants less attractive to the pest insects.

The majority of wild plants in nature and agricultural crop plants depend on cross-pollination by pollinators for seed-set and the production of fruits. In this respect, two important setbacks have been recognized recently. Firstly, the biodiversity of pollinating insects has been shown to decline alarmingly, threatening the maintenance of biodiversity of wild plants, and causing a decrease of pollination efficiency of field-grown agricultural crops (Osborne *et al.*, 1991). Secondly, in plant breeding programs elite cultivars are selected that express valuable characters, e.g. disease resistance or hybrid seed production. However, in field trials, these plants very often appear unattractive to pollinating insects.

Nectaries are the nectar-secreting glands of flowers, and nectar is the main floral reward for pollinating insects. So, nectar quality is the key factor, which can be used to select plants that are more attractive to pollinators. The new biotechnological approaches to understand genetic regulation of nectary development and to modify nectar composition will make it feasible to change the sugar type and sugar concentration of nectar. This will lead to the production of plants that are more attractive to pollinators.

In this thesis, we used a model plant species *Petunia hybrida* and isolated a novel nectary-specific gene, *NEC1*. The *NEC1* gene and its promoter were characterized, and the gene was found to be highly expressed in the nectaries and in specific parts of the stamens of *Petunia hybrida*. Our finding that *NEC1* expression was only observed in the nectar-secreting lateral nectaries of *Brassica napus*, indicates that the *NEC1* gene is involved in the regulation of nectar production. From studies on the function of the gene, it was shown that *NEC1* plays an important role in sugar metabolism and nectar production. Its presumed role is in the initiation of the hydrolysis of starch, which accumulates in nectary parechyma cells up to the stage of anthesis. By the expression of *NEC1*, starting in the outer nectary cells, starch is degraded to hexoses. The released monosaccharides build up an osmotic gradient to activate nectar secretion and they also contribute substantially to nectar production. In addition, the formation of nectaries was prevented by transformation with a *NEC1*-RNase construct.

Using nectary-specific genes or promoters, it might be possible to genetically modify plants in such a way that they can produce pharmaceutical proteins in nectar. Honey produced from such plants could, thus contain the medicinal protein. In view of the sugar metabolism genes, cloned from several

plant species (Frommer and Sonnewald, 1995; Lerchi *et al.*, 1995), the prospects for the application of genes that modify nectary development or nectar production are discussed in this chapter.

Enhancing biodiversity of pollinating insects and wild plants

The habitats of many species of wild flowering plants and bees are declining (Osborne *et al.*, 1991). Large parts of Mediterranean shrub-lands that traditionally form a very good habitat for pollinating insects, have been lost by overgrazing, frequent fires, urbanization, and tourism (Petanidou and Smets, 1995). Simultaneously, pollinator habitats have been lost due to the extensive practices of agriculture. The decline of biodiversity of native pollinators and wild flowers has emphasized the necessity for a well-designed management of semi-natural lands.

Selection and modification of plants with high nectar quality, i.e. semi-natural lands will counteract the decline of biodiversity of pollinating insects. An environmentally friendly exploitation of such areas can be achieved by the establishment of balanced vegetation of flowering plants that can restore the biodiversity of pollinating insects. An environmentally friendly on-farm activity that will benefit largely from such a policy, is apiculture. Based on our present knowledge, it is possible to use nectary-specific promoters to express the sugar metabolism genes (Frommer and Sonnewald, 1995; Lerchi *et al.*, 1995) in nectaries to change only the sugar type and concentration of the nectar, and to investigate its effects on pollinator attraction. Considering the nectar and nectary parameters of different varieties or semi-natural crops correlated with high or low values for pollinator attractiveness, this knowledge will form the scientific basis to select plants that are more attractive to pollinators for use of *semi-natural* crops in nature management programs.

Increasing flower attractiveness and improving pollination efficiency

Pollination is a very important process in plant breeding and agriculture as well as in horticulture. However, inadequate pollination is one of the major problems in seed production, especially in hybrid seeds. Nectar quality is an almost totally neglected trait in plant breeding programs. The evident problem is that in hybrid seed programs the selected parental lines are often unequally attractive to foraging insects. Thus, it results in poor cross-pollination and consequently low hybrid seed production (Pham-Delegue *et al.*, 1991).

As is known, cotton (*Gossypium spp.*) is an economically important and extensively grown crop. Cotton is usually referred to as a partially cross-pollinated crop. The benefits derived by cotton from insect pollination, in terms of greater and

higher quality lint and seed production, earliness of harvest, better germination and improved qualities in the offspring, have been cited by numerous workers. Especially hybrid cotton plants show high quality and quantity of production because of hybrid vigor. In large-scale production of true hybrid seeds, flowers of the male sterile line need to be pollinated by male fertile lines. However, cotton pollen is too heavy and sticky to be carried by wind and has to be transferred either by hand or by pollinating insects. Insect pollination is preferred to effect cross-pollination but is hampered, because cotton flowers are unattractive to honeybees.

Cotton has five sets of nectaries, one floral and four extrafloral (Free, 1970; McGregor, 1976). Nectar is secreted from floral nectaries as well as from extrafloral nectaries and the volume of floral nectar exceeds that of extra-floral nectar. Cotton flowers have a long flowering period and contain large amounts of nectar and pollen, being an excellent crop for honey quality. However, honeybees appear to be noticeably reluctant to visit cotton blossoms. In addition, honeybees have been shown to prefer extra-floral nectar above floral nectar, and are often reluctant to enter the cotton flower. Foraging on extra-floral nectar is undesirable, as it does not effect cross-pollination. Moreover, honeybees have been shown to forage more frequently on male sterile flowers than on male fertile flowers in cotton species used for hybrid seed production. The overall, behavior of honeybees towards cotton flowers thus results in poor cross-pollination and seed production.

Floral nectar of upland cotton has been analyzed by gas chromatography (Butler *et al.*, 1972). It contains very low quantities of sucrose (approximately 7%) and equal amounts of glucose and fructose (approximately 37%). It was shown by Wykes (1952b) that honeybees prefer balanced nectars that contain equal ratios of fructose, glucose and sucrose. Furthermore, honeybees prefer sugar concentrations of at least 30 to 50 percent (Waller, 1972). The low percentage of sucrose in floral nectar may be the reason that cotton flowers are unattractive to honeybees when other nectars are available (Butler *et al.*, 1972).

Genetic modification of plant nectar to control pest insects

Extrafloral nectaries undoubtedly serve a useful biological function, e.g. to attract beneficial insects that prey on pest insects (Rogers, 1985). However, they seem to have a detrimental effect on pollination, especially when they secrete nectar before the flowers open. This is because the pollinators that become conditioned to collect extrafloral nectar do not visit the flowers any more and, therefore, do not affect pollination (Free, 1970). Nectar secreted from the extrafloral nectaries attracts pests, such as cotton aphids. Extrafloral nectaries are important routes of entry for larvae which damage ovaries of cotton squares (Belcher *et al.*, 1984). Some authors investigated the effects of removal of extrafloral nectaries on insect control (Peacock *et al.*, 1996). Breeding of lines that have recessive genes, ne1 and ne2,

results in cotton plants that have no extrafloral nectaries on the leaf midribs, the bracts and between the bracts and the calyx. The nectariless trait indeed reduced the incidence of pest insects on cotton plants. However, the authors did not discuss the effect on honeybees and on beneficial insects that prey on pest insects (Maxwell *et al.*, 1976). E.g., in pest management, cotton genotypes that lack extrafloral nectaries have been selected which are resistant to *lepidopterous* pests. But the use of these cultivars can also have a serious impact on the natural enemies of pest insects. It might be possible to select or genetically modify crop genotypes having extrafloral nectar that is unpalatable to pests, but is still attractive to entomophagous or pollinating insects (Rogers, 1985).

It is a new approach to combine nectar secretion genes with *Bt* (*Bacillus thuringiensis*) genes to improve plant resistance to pest insects. The technology for obtaining transgenic plants resistant to pest insects is available for almost all the important cultivated plants or crop species. The use of *Bt* toxin genes is widely known as a model for genetically engineered insect resistance (Whitten *et al.*, 1996). Different toxins, produced by different bacterial strains, show a high degree of specificity. *Lepidoptera*-, *Coleoptera*-, *Diptera*- and *Nematode*- specific proteins have been found, and the list is still increasing with time (Holsters *et al.*, 1993; Mandaokar *et al.*, 1999). The *Bt* gene confers resistance to some of the most destructive pest insects in transgenic crops such as maize, potato and cotton (Carozzi and Koziel, 1997). When honeybee colonies were fed with sugar syrup from pollen obtained from *Bt* (CryIIIB)-transgenic plants, containing a 2000 times higher protein content, no toxic effects on larvae were observed (Arpaia, 1996). Meanwhile, improvement of plant attractiveness to the pollinators could be achieved by genetic modification of nectar composition. It will lead to an important break through in agricultural sciences if nectar composition can modified in order to make transgenic flowers more attractive to pollinators, e.g. bees and plants less attractive to pest insects.

CHAPTER 7

SUMMARY AND CONCLUDING REMARKS:

SUMMARY

SUMMARY IN DUTCH (SAMENVATTING)

SUMMARY IN CHINESE

Summary

Nectaries are the nectar-secreting glands of flowers and they uniquely function as a floral reward for pollinating insects (chapter 1). Various plant species and varieties differ with respect to the location and structure of nectaries as well as to the composition of the nectar. This variation has evolved during co-evolution of plant species and their pollinating insects, thus resulting in unique plant-pollinator relationships (chapter 2). In plant breeding programs little attention is paid to the trait of nectar quality, consequently, many crop plants turn out to be unattractive to pollinating insects (chapter 6). Nectar quality is the key value that can be used to select or modify plants in such a way that they are more attractive to pollinators. A new biotechnological approach is discussed to modify nectar composition through the use of nectary-specific promoters (chapter 6).

The primary goal of the research presented in this thesis was to identify and characterize genes, ultimately responsible for nectar production and nectary development in *Petunia hybrida*. The work described in this thesis was undertaken by a molecular approach using differential display RT-PCR. We successfully cloned and characterized a nectary-specific gene, *NEC1* (chapter 3).

The full-length cDNA of *NEC1* is 1204bp encoding a protein of 265 amino acid residues (chapter 3). The gene structure consists of six exons and five introns, spanning a region of about 2.2kb (chapter 4). The secondary structure of the putative NEC1 protein is reminiscent of a transmembrane protein, indicating that the protein is incorporated in the cell membrane or the cytoplast membrane. The putative NEC1 protein shows high homology (47% identity, 72% similarity) with *MtN3*, a *Rhizobium*-induced gene that is involved in nodulation in the legume *Medicago trunculata* (chapter 3). Southern blot and sequencing analyses indicated the presence of two highly homologous genes: *NEC1* and a *NEC1*-like gene, *NEC2* in the *Petunia* genome (chapter 4).

Immunolocalization using a peptide antibody revealed that NEC1 protein is detected mainly in the outer nectary parenchyma cells. Northern blot and RT-PCR analyses showed that *NEC1* is highly expressed in the nectary tissue and weakly in the stamen. GUS expression driven by the *NEC1* promoter revealed GUS activity in the nectary parenchyma cells, the upper part of the anther filament and the anther stomium. GUS expression is detectable as blue spots on the surface of nectaries from very young flower buds (1.7 cm) and increased during further floral and nectary development. GUS expression is the highest in nectaries of opened flowers during anthesis that actively secrete nectar and is then present in all the nectary parenchyma cells. Ectopic expression of *NEC1* resulted in transgenic plants that displayed a phenotype with leaves having 3-4 times more phloem bundles in the mid veins compared to wild type *Petunia* (chapter 3).

To further elucidate the function of *NEC1*, co-suppression was performed to achieve down-regulation of *NEC1* expression, and transposon insertion mutagenesis was used to knock out the *NEC1* gene function. Only partial gene silencing of *NEC1* realized, which did not result in a nectary phenotype but did show an interesting phenotype in the anthers. In this phenotype, termed "early open anther", anthers already open in young flower buds (1.8cm) that still contain immature pollen, resulting in poor pollen quality and impaired pollen release. The results obtained reveal that *NEC1* might be involved in the development of stomium cells, which are ruptured during the normal process of anther dehiscence to release mature pollen (chapter 4).

Complete down-regulation of *NEC1* gene expression was achieved when *NEC1* antisense was introduced under control of the *NEC1* promoter. Transgenic plants showed a distinguishable nectary phenotype with defects in the outer nectary parenchyma cells. Nectary development was impaired and nectar secretion was strongly reduced. The "early open anther" phenotype was also observed in these transgenic plants. Considering that *NEC1* is only expressed in the nectar-secreting lateral nectaries of *Brassica napus* (chapter 3), the results reveal that *NEC1* plays a role in nectar production and nectar secretion. Its presumed role is in the initiation of the hydrolysis of starch, which accumulates in nectary parechyma cells up to the stage of anthesis. By the expression of *NEC1*, starting in the outer nectary cells, starch is degraded to hexoses. The released monosaccharides build up an osmotic gradient to activate nectar secretion and they also contribute substantially to nectar production (chapter3 and 5). Furthermore, for the first time a nectariless phenotype was obtained by a nectary targeted cell ablation by *Barnase* (chapter 5).

The prospects for application of genes that regulate nectary development and nectar production are discussed in chapter 6.

In conclusion, *NEC1*, a novel gene, is highly expressed in nectaries and is also expressed in specific cells of the stamen. Functional analyses of *NEC1* by over-expression, co-suppression, transposon insertion mutagenesis and antisense down-regulation reveal that *NEC1* displays a very timed and restricted function in two floral developmental processes, nectar secretion and anthesis. *NEC1* presumably function as an osmoregulator by timing the hydrolysis of starch in specific tissues of the nectaries and the anthers. Concomitantly, *NEC1* plays a major role in nectar production by the release of hexoses from starch in nectaries.

Samenvatting en conclusies

Nectar is het zoete vocht dat insecten uit bloemen halen als 'beloning' voor de bestuiving. Het wordt uitgescheiden door de nectariën (hoofdstuk 1). Zowel de samenstelling van de nectar als de plaats en vorm van de nectariën is verschillend tussen soorten en variëteiten. Deze variatie is onstaan tijdens de co-evolutie van plantensoorten en hun bestuivers, waardoor een unieke plant-bestuiver relatie is ontstaan (hoofdstuk 2).

In veredelingsprogramma's worden 'elite cultivars' geselecteerd op waardevolle kenmerken zoals ziekteresistentie of productie van veel zaad. Helaas zijn zulke uitgeselecteerde rassen vaak niet attractief voor bestuivende insecten (hoofdstuk 6). Een nieuwe biotechnologische benadering, om planten op basis van de nectar kwaliteit te selecteren op hun aantrekkingskracht op insecten, is het controleren van de genetische regulatie van de ontwikkeling van nectariën en het modificeren van de nectarsamenstelling met behulp van nectariën-specifieke promoters (hoofdstuk 6). Een promoter is een stukje DNA dat gelegen is voor de genetische informatie van een eiwit en dat er voor zorgt dat het eiwit alleen in de juiste cellen en op de juiste tijd wordt aangemaakt. Bij deze benadering wordt gebruik gemaakt van transgene planten. Bij transformatie van planten wordt een extra stukje genetisch materiaal ingebracht dat codeert voor de te onderzoeken functie (een eiwit).

Het hoofddoel van het onderzoek, dat in dit proefschrift wordt gepresenteerd, is de identificatie en karakterisering van genen die verantwoordelijk zijn voor de ontwikkeling van nectariën en voor de productie van nectar in *Petunia hybrida*. Het onderzoek dat hier wordt beschreven is uitgevoerd via een moleculaire aanpak waarbij gebruik is gemaakt van de zgn. 'differential display RT-PCR' techniek. Hiermee is het gelukt om een nectariën-specifiek gen, *NEC1* te kloneren en te karakteriseren (hoofdstuk 6).

De totale lengte van het cDNA van *NEC1* is 1204 basenparen (bp) die coderen voor een eiwit van 265 aminozuren (hoofdstuk 3). Het gen bestaat uit zes exons en vijf introns die een gebied omvatten van 2.2 kb (hoofdstuk 4). De afgeleide structuur van het veronderstelde NEC1 eiwit komt overeen met die van een transmembraaneiwit hetgeen erop duidt dat het eiwit is opgenomen in de celmembraan of in de cytoplastmembraan. Het veronderstelde NEC1 eiwit heeft een grote homologie (47% identiek, 72% overeenkomstig) met *MtN3*, een gen dat door *Rhizobium* wordt geïnduceerd en dat betrokken is bij de wortelknol vorming in *Medicago trunculata* (hoofdstuk 3). Uit Southern blotting en sequentie analyse is gebleken dat er in het genoom van *Petunia* twee zeer sterk overeenkomstige genen zijn: *NEC1* en *NEC2* (een *NEC1*-achtig gen).

Met behulp van 'Northern blots' en 'RT-PCR' is vastgesteld dat het gen sterk actief is in het nectariën-weefsel en zwak in de helmhokjes. Met 'immunolocalisatie', waarbij gebruik is gemaakt van een antilichaam tegen het

eiwit, is vastgesteld dat het NEC1 eiwit voornamelijk voorkomt in de buitenste laag parenchym cellen van het nectariën-weefsel. Met behulp van de expressie van een markeringseiwit, GUS, onder controle van de *NEC1* promoter, kon de gen activiteit nader worden gespecificeerd en gelocaliseerd in de parenchymcellen van de nectariën, in het bovenste deel van het filament en in het stomium van de helmhokjes. De GUS expressie is zichtbaar als blauwe vlekken op het oppervlak van de nectariën op bloemknoppen vanaf 1.7 cm en neemt toe tijdens de verdere ontwikkeling. De hoogste GUS activiteit is gevonden in nectariën tijdens het open gaan van de helmhokjes (de anthese) in open bloemen die actief nectar produceren. In dat stadium komt het voor in alle parenchymcellen. Transgene *Petunia* planten, waarin *NEC1* buiten de nectariën (ectopisch) tot expressie wordt gebracht, hebben een fenotype waarbij in de bladeren het aantal zeefvaten in de centrale vaatbundel 3 tot 4 keer zo hoog is als in wild type planten (hoofdstuk 3).

Om de functie van *NEC1* op te helderen is met behulp van 'co-suppressie' de *NEC1* expressie sterk verlaagd terwijl met transposon mutagenese geprobeerd is het gen geheel uit te schakelen ('gene silencing'). Hiermee kon het gen slechts gedeeltelijk worden stilgelegd en het resulteerde in het vervroegd open gaan van de helmhokjes. In dit fenotype, dat 'early open anthers' is genoemd, gaan de helmhokjes al open in jonge knoppen (1.8cm) die nog onvolgroeide pollenkorrels bevatten. Dit resulteert in een slechte kwaliteit pollen die niet goed vrijkomen. Uit deze waarnemingen kan geconcludeerd worden dat *NEC1* betrokken zou kunnen zijn bij de ontwikkeling van de stomium cellen. Bij bij de normale ontwikkeling scheuren de stomium cellen open waardoor de helmhokjes open springen en het rijpe pollen vrij kan komen (hoofdstuk 4).

Een volledige uitschakeling van de *NEC1* genexpressie kon worden verkregen door *NEC1* in een 'antisense' oriëntatie onder controle van zijn eigen promoter te introduceren in *Petunia* planten. De aldus verkregen transgene planten hadden een duidelijk nectariën-fenotype met afwijkingen in de buitenste parenchym cellen waarbij de ontwikkeling was gestoord en de nectar productie sterk was verminderd. Deze planten vertoonden ook het 'early open anther' fenotype. Rekening houdend met het feit dat in *Brassica napus NEC1* alleen in de laterale nectariën tot expressie komt (hoofdstuk 3) kunnen we concluderen dat *NEC1* betrokken is bij de productie en secretie van nectar. Hierbij volgt de expressie het proces van zetmeel afbraak in de tijd. In de transgene planten is de zetmeel afbraak geremd waardoor er een lagere osmotische druk ontstaat in de cellen van de nectariën. Dit duidt erop dat NEC1 vermoedelijk als osmoregulator betrokken is bij de nectar productie en secretie(hoofdstukken 3 en 5).

In hoofdstuk 5 is tevens beschreven dat het voor de eerste keer is gelukt om planten met een fenotype zonder nectariën te verkrijgen door gebruik te maken van een specifiek op nectariën gerichte cel eliminatie met het transgen *Barnase*.

De verschillende voorbeelden uit dit proefschrift om de nectariën ontwikkeling en nectar secretie te modificeren leiden tot te verwachten

toepassingen in de plantenveredeling. Deze toekomstverwachtingen zijn beschreven in hoofdstuk 6.

Concluderend, *NEC1* is een nieuw geïdentificeerd en geïsoleerd gen dat hoog tot expressie komt in nectariën, maar ook in de stomium cellen en het filament. Uit de functionele analyse middels verlaging of uitschakeling van de genexpressie door over-expressie, co-suppressie, transposon insertie mutagenese en antisense technologie is gebleken dat *NEC1* een zeer specifieke functie heeft in twee ontwikkelingsprocessen van de bloem, namelijk de uitscheiding van nectar en het openspringen van de helmhokjes. De rol van NEC1 bij de nectar productie en excretie is waarschijnlijk als osmoregulator.

NEC1，矮牵牛 (*Petunia hybrida*) 的蜜腺发育和花蜜合成的调节基因

(中文摘要)

葛亚新

蜜腺 (Nectary) 是植物分泌蜜液 (Nectar) 的主要器官及组织，其分为花内和花外蜜腺两种。花内蜜腺位于花内，而花外蜜腺则主要位于植物的营养器官如叶子、茎上等。在植物和昆虫的长期共同进化过程中，蜜腺的形成使植物与授粉昆虫之间建立了特有的互惠关系－植物分泌的蜜液为昆虫提供了食物和能量，昆虫的授粉辅助植物完成其有性生殖过程。花内蜜腺分泌的蜜液 (花蜜)，担负着吸引昆虫到花上来采蜜授粉的职责，花蜜的质量和成份是决定植物的花是否吸引授粉昆虫如蜜蜂的关键因素。

到目前为止，尽管已有了很多的关于蜜腺的形态、结构、发育和授粉生物学方面的研究报道，但关于蜜腺发育的遗传控制及其基因功能的研究很少。本论文综合报道了从矮牵牛 (*Petunia hybrida*) 蜜腺中分离蜜腺特异性基因 *NEC1* 及其功能分析的研究结果。

矮牵牛有一对花内蜜腺连成环状，位于子房的基部。*NEC1*基因是通过差异显示 (Differential Display RT-PCR) 的分子生物学技术从矮牵牛的蜜腺中分离到的蜜腺特异基因。*NEC1* 的 cDNA 全长为 1204 个碱基对，编码具有 265 个氨基酸的蛋白质。核基因是由六个外显子和五个内含子构成，总长为 2200个碱基对。NEC1 蛋白质是一种膜蛋白，其结构和豆科的固氮根瘤诱导基因 *MtN3* 有很高的同源性。Southern 杂交和基因序列分析表明在矮牵牛的基因组中除了 *NEC1* 基因外，还存在一个与其*NEC1* 高度同源的*NEC2* 基因。

Northern 杂交和 RT-PCR 实验表明 *NEC1* 基因在蜜腺组织中高度表达，在花药上则有微弱的表达。由 *NEC1* 启动子带动的GUS 基因显示GUS 活力主要位于蜜腺的薄壁细胞，花药的开裂口及花丝的顶部。*NEC1* 基因表达在长 1.7cm 以上的花蕾中可以观察到。随着花和蜜腺的进一步发育，花蜜的分泌增加，*NEC1* 的表达活力也随着逐渐增强。在开花期，花蜜分泌最为旺盛，*NEC1* 基因的表达也最为活跃。该结果表明

NEC1 基因表达可能和蜜腺内的淀粉降解和糖份的累积有关，当足够多的糖份分泌到蜜腺外就形成了蜜液。有趣的是当我们把同样的基因构件转化到油菜 (*Brassica napus*) 时，*NEC1* 活性只在转基因植株的与分泌有关的侧蜜腺 (Lateral Nectary) 上特异表达，而在与分泌无关的主蜜腺 (Median Nectary) 上不表达，间接证明了 *NEC1* 基因和蜜液的合成及分泌有关。通过 NEC1 抗体的免疫定位分析 (Immunolocalization) 证明 NEC1 蛋白质主要分布在蜜腺薄壁细胞的质膜周围，并在此观察到了囊泡与质膜融合的分泌现象。

为了进一步分析 *NEC1* 基因的功能，首先将构建的抑制 *NEC1* 表达的基因构件 (Co-suppression)转化到矮牵牛中。由此得到的转基因植株中 *NEC1* 基因表达受到了抑制，表现出了“早裂花药”现象。在正常的矮牵牛植株上，花蕾长到 6 cm 以上时花瓣完全展开，在开花期花粉粒发育成熟，花药正常开裂。“早裂花药”现象是指当花蕾只有 1.8 cm 大时，花药就开裂了，此时花粉粒还远没有成熟，甚至裂口细胞还没有完全形成。另外，从 2410 株带有活跃的 *dTph1* 转座子(*dTph1* transposon element) 群体中筛选出了一株 *dTph1* 插入到 *NEC1* 基因的突变株。该突变株也表现“早裂花药”现象。但这两种途径获得的变异株都没有观察到蜜腺的变化。本研究认为这是因为当 *dTph1* 插入到 *NEC1* 基因时，只抑制了 *NEC1* 基因的表达，而 *NEC2* 基因仍很活跃并行使着类似的功能，Northern杂交分析结果表明 *NEC* 基因的 RNA 转录水平只受到了部分抑制也证明了这一点。由于花药裂口细胞的发育需要更多的 *NEC1* 基因产物，当 *NEC1* 基因转录受阻时，*NEC2* 基因产物不能完全满足裂口细胞发育的需要，从而导致了“早裂花药”现象。另外，不同学者对抑制基因表达构件的启动子的专一性和强度的要求持不同的看法。由于 *NEC1* 抑制表达的基因构件是由 花椰菜花叶病毒 (CaMV) 35 S 外壳蛋白的启动子(称为 35 S 启动子) 驱使的，而 35 S 启动子在蜜腺上并不表达，本研究认为若要完全抑制 *NEC1* 基因在蜜腺上的表达，有必要采用在蜜腺特异表达的启动子。

进一步将 *NEC1* 启动子驱使的 *NEC1* 反意抑制表达构件 (Antisense inhibition) 转化到了矮牵牛上，由此得到的转基因植株的蜜腺发育受阻，表现在蜜腺外部的薄壁细胞的发育退化及蜜液分泌的明显减少和完全没有。在这些变异的转基因植株上，也观察到了“早裂花药”现象。另外，在转基因植株上都观察到了蜜腺细胞和花药的裂口细胞受到糖份胁迫导致的褐变现象。该结果，进一步证明了 *NEC1* 与糖份的代谢

和累积有关，在蜜腺的发育、蜜液的合成及分泌和花药的裂口细胞的形成过程中起着很重要的作用。当 *NEC1* 基因在矮牵牛上过份表达(Over-expression) 时，转基因植株主叶脉的维管束数目比正常的对照高出 3 - 4 倍，维管束是植物糖份转移的主要通道。

综上所述，我们从矮牵牛的蜜腺中分离到的 *NEC1* 基因，在蜜腺和花药的裂口细胞上表达。该基因可能在花发育的蜜液分泌和花药开裂的两个重要过程中起着糖份代谢的渗透调节作用。

另外，本文还首次报道了由 *NEC1* 启动子驱动细菌的细胞致死基因 *Barnase* 的表达，由此得到了无蜜腺的转基因植株。这对非昆虫授粉作物，特别是一些观赏植物的抗虫能力的提高有着深远的意义。目前，在本研究的基础上荷兰的 **Plant Research International (PRI)** 研究所开展了与应用有关的两个新研究方向：一个是利用蜜腺特异性启动子驱动从不同物种分离到的控制糖份代谢有关基因的表达，研究这些基因对蜜腺发育和蜜液成份的影响。二是利用蜜腺特异性启动子驱动一些特定的药用蛋白质合成基因在蜜腺中表达，合成含有药用蛋白的蜜液，经蜜蜂采蜜，药用蛋白在蜜蜂体内得到了浓缩后，随后被累积在高糖成份的蜂蜜中，既便于储藏又便于进一步的纯化。此外，一些疫苗的试制工作也正在进行之中。因此，蜂蜜药物已不再只是个设想，正在变为现实。

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Curriculum Vitae

Ge Yaxin was born on November 10, 1959 in Hebei province, China. She completed her B.Sc. study at the Department of Agronomy, Beijing Agricultural University (now China Agricultural University) in 1982. Afterwards, she worked as an assistant teacher in Zhejiang Agricultural University (now Zhejiang University) from 1982 to 1984. After a three years study in Plant Genetics and Breeding at Beijing Agricultural University, she received her M.Sc. degree in 1987. Then she became a research scientist at Hebei Academy of Agricultural and Forestry Sciences. Her scientific and technical skills were further improved by working as a visiting scientist for 8 months in the former Station for Plant Breeding (SVP) in Wageningen. In 1995, she obtained a Marie Curie grant from the EU and an IAC fellowship, which enabled her to work for two years as a visiting scientist in the Department of Developmental Biology, CPRO-DLO (now Business Unit Plant Development and Reproduction, Plant Research International). During this stay, she initiated her Ph.D. study, from 1997-2001, at the Business Unit Plant Development and Reproduction, Plant Research International, Wageningen University and Research Center, in cooperation with the Department of Molecular Plant Physiology, the University of Nijmegen (Prof. Dr. George J. Wullems). From January 2001, she is employed at The Samuel Roberts Nobel Foundation, Inc., Ardmore, Oklahoma in USA and carries out research on forage biotechnology.

This thesis describes an efficient cloning, characterization and functional analysis of a nectary-specific gene in *Petunia hybrida*. *NEC1*, a novel gene, is highly expressed in nectaries. *NEC1* is involved in nectar production and nectar secretion. Nectar secreted from floral nectaries is the main floral reward for pollinating insects and nectar quality is the key value that can be used to select plants that are more attractive to pollinators. Genetic regulation of nectary development and modification of nectar composition will allow to make the flowers more attractive to pollinators, and the plants less attractive to pest insects.